REMARKS/ARGUMENTS

Claims 1-16 are pending in the present application. In a Communication filed July 28, 2005, Applicants elected the Group I claims, nos. 1-14 and 16, drawn to a complex immuno-gene medical composition for inhibiting tumor cells, comprising DNA Sequences SEQ ID NO:1 and SEQ ID NO:2, and a method of using the composition to inhibit the growth of tumor cells, wherein CTVT was further elected as the species of tumor cells and muscle electroporation was elected as the species of plasmid administration. In the presently pending Office Action concerning this application, claims 1-14 and 16 are rejected and claim 15 is withdrawn from consideration by the Examiner as being directed to a non-elected invention. In response to these rejections, claims 1-16 have been canceled without prejudice or disclaimer and replaced by new claims 17-27 which more clearly define the invention elected in applicants' July 28, 2005 Response. These new claims are entirely supported by the application as originally filed and thus they add no new matter. Entry of the new claims into the file of the application is, therefore, respectfully solicited.

OBJECTIONS TO THE SPECIFICATION

The Examiner objects to the specification as not being written in, "full, clear, concise and exact terms" as required under 35 U.S.C. §112, First Paragraph. He states that the specification is replete with terms which are not clear, concise and exact. The Examiner, therefore, requires the applicants to provide a fully corrected specification, without introducing new matter.

In response, applicants submit that although they believe that one of ordinary skill in this art would adequately comprehend their invention based on the teachings contained in the application as originally filed, in the interest of advancing the prosecution of this application they are providing herewith two attachments responsive to the objections raised by the Examiner. The first attachment, i.e, **Exhibit A**, is a 'marked-up' copy of the specification as filed, indicating numerous formal (e.g., grammatical, spelling, etc.) changes to the text which are believed to render the specification more understandable. It is, however, believed that these changes do not diminish to any degree the scope of the invention originally described therein by the applicants. The second attachment (**Exhibit B**) is a 'clean copy' of the specification containing all of the proposed changes set forth in the mark-up. None of the proposed changes to the specification are believed to add any new matter. The Examiner is, therefore, respectfully requested to enter the 'clean' specification, as amended, into the file of this application and to reconsider and withdraw the objections to the specification recited in the Office Action.

CLAIM OBJECTIONS

Claims 1-14 and 16 are objected to due to several alleged informalities as described on p. 3 of the Office Action. The Examiner states that the claims contain multiple grammatical errors and misspellings.

In response, as noted above, the objected-to claims, as well as withdrawn claim 15, are all canceled herein without prejudice or disclaimer and replaced by new claims 17-27 which more clearly express the invention which applicants seek to patent. These new claims are written in a form which, it is believed, meets all of the statutory requirements for claiming the invention. The Examiner is therefore respectfully requested to enter these new claims and to reconsider and withdraw the claim objections as described on p. 3 of the Office Action.

CLAIM REJECTIONS UNDER 35 U.S.C. 112

Rejections Under 35 U.S.C. §112, First Paragraph

Claims 1-14 and 16 are rejected under 35 U.S.C. §112, First Paragraph because, according to the Office Action, while the specification is enabling for a therapeutic composition for inhibiting Canine transmissible venereal tumor (CTVT) cells, comprising a plasmid comprising SEQ ID NO:1 encoding IL-6, and a plasmid comprising SEQ ID NO:4 comprising an IL-2 signal peptide operably linked to IL-15, and a method of inhibiting the growth of CTVT tumor cells in CB-17 SCID mice, *in situ*, by administering the plasmids via muscle electroporation, the specification does **not** reasonably provide enablement for a therapeutic composition for inhibiting **any** tumor cells, comprising a plasmid comprising SEQ ID NO:1 encoding IL-6 and a plasmid comprising SEQ ID NO:2 encoding IL-15, and a method of inhibiting the growth of **any** tumor cells in CB-17 SCID mice, *in situ*, by administering the plasmids via muscle electroporation. Further according to the Examiner, "The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with those claims. The bases for the Examiner's rejections are set forth on pps. 4-9 of the Office Action. The §112, ¶1 rejection is respectfully traversed for the reasons set forth below

In response, *inter alia*, to the rejection under §112, First Paragraph, applicants have, as noted above, canceled without prejudice or disclaimer original claims 1-16 and replaced them with new claims 17-27. In this new claim set, claims 17 and 22 are the only claims written in independent form. New claim 17 recites a complex immuno-gene medical composition. As indicated in the claim, the composition is not adapted for use in inhibiting any tumor cells; rather, the claim specifically recites that the composition is for inhibiting (only) "tumor cells producing TGF-β". In

like manner, claim 22 is directed to a method of inhibiting not 'any' tumor cells, but instead only those tumor cells "producing TGF-β ". The recitation of this feature in the proposed new set of claims is supported by the specification as originally filed and thus it adds no new matter. Applicants submit that their specification is believed to clearly enable claims of the scope now proposed, and thus the §112 'non-enablement' rejection of original claims 1-14 and 16 should be withdrawn.

Further to the above, the following additional comments are provided in response to the Examiner's remarks on pps. 4-9 of the Office Action in support of his 'non-enablement' rejection of claims 1-14 and 16.

The Examiner states, in the paragraph bridging pps. 4-5 of the Office Action, that no other plasmids, i.e, than a first plasmid comprising a sequence encoding IL-6 (SEQ ID NO:1) and a second plasmid comprising a sequence encoding the human IL-2 signal peptide (IL-2SP operably linked to a sequence encoding the mature human IL-15 peptide, which forms a chimeric IL-2 SP/IL-15 gene (SEQ ID NO:4), are disclosed by applicants' specification. However, as regards the enablement of the set of new claims provided herewith, the Examiner's attention is respectfully directed to p.9, para. [0032], on lines1-3, wherein the specification teaches that " Commercial pcDNA3.1/V5-His-TOPO TA Expression Kit is applied to clone and construct the plasmids containing IL-6 gene and IL-2 SP/IL-15 MP chimeric gene, respectively.". New claim 21 recites that the plasmid of (independent) claim 17 may be a pcDNA3.1/V5-His-TOPO vector and thus the subject claim is enabled by the teachings contained in the above-mentioned para. [0032].

In addition, p.9, paragraph [0032], lines 1-3 state that, "Commercial pcDNA3.1/V5-His-TOPO TA Expression Kit is applied to clone and construct the plasmids containing IL-6 gene and IL-2 SP/IL-15 MP chimeric gene, respectively.". Thus the plasmid in new independent method claim 22 is limited to a pcDNA3.1/V5-His-TOPO vector. And as further recited in the subject claim, the administrating step is carried out via muscle electroporation in vivo, which is the mode of administration elected by the applicants in their previous response submitted July 28, 2005.

The Examiner states on p. 5 of the Office Action that the working examples in the specification do not indicate what the average phase of the CTVT cells transplanted into the mice were. In response, applicants submit that FIG. 6 and FIG. 7 illustrate that the tumor volume in the group treated with a Mock vector shows a dramatically linear increasing tendency which does not plateau or decrease. Those Figures evidence that the CTVT cells transplanted into the mice within

the experimental period are in the P phase but not the S or R phase. Therefore, the tumor reduction effect shown in the indicated Figures does not represent the R phase of the CTVT. Instead, the results set forth therein are clearly attributable to the treatments with the complex immuno-gene medical composition of the present invention, i.e, as recited in claims 17 and 22.

The Examiner states further on pp. 5-6 of the Office Action that, "The basic premise of the claimed invention is that the NK cells have been upregulated in the SCID mouse model . . . However, the working examples do not analyze or indicate what effect these cytokines have on the CTVT cells.....such as ILT-2, which recognize non-MHC ligands such as UL18" (see Office action, page 5, line 20 to page 6, line 10). In response, the applicant submits as follows:

According to the description of the invention found on p. 2, paragraph [0006], lines 1 to 3; p.2, paragraph [0005] and p.1, paragraph [0003], lines 3 to 6 of the present specification, the cytotoxicity of mice NK cells against xenogeneical tumors can be explained by the classical term "missing self" (Ljunggreb, HG and Karre K, In search of the "missing self" : MHC molecules and NK cell recognition. Immunol. Today 11:237-244, 1990).

Missing self implies that, when NK cells can not detect self MHC on a cell, the inhibitory receptors on NK cell are quiescent. In contrast, however, the activating receptors are working so that the activated NK cells are able to kill the target cells that lack self MHC.

The medical composition of the present application can restore the "missing self" mechanism of NK cells to kill the target cells expressing large amount of TGF-β, such as CTVT tumor cells. The present medical composition is used to promote the cytotoxicity of NK cells or to elevate the percentage of NK cells, but it does not directly work on the target cells.

The mechanisms on the molecular level in CTVT cells are, at present, still not entirely clear to workers in this field. Moreover, MICA and MICB are non-classical MHC produced in humans, related to NK activity in inflammatory reaction or viral infection, and are not usually associated with cancer cells. The description in the specification clearly supports the subject matter in the new claims, and complies with 35 U.S. C. 112 in that it would enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with these claims even though no evidence is provided regarding how the reactions operate on a molecular level.

Further to the above, in comparing the groups treated with the Mock vector with those treated with the combination of the IL-6 plasmid and IL-15 plasmid, Figs 6-9 clearly demonstrate that the treatment with the combination of the IL-6 plasmid and IL-15 plasmid significantly reduces tumor size and results in a higher survival percentage. However, when the anti-asialo GM-1

antibody is employed in the treatment to block the function of NK cells, no tumor reduction effect is observed. On the other hand, FIG. 4 (C) also demonstrates that the combined usage of IL-6 plasmid and IL-15 plasmid can significantly elevate the percentage of NK cells. Also, FIG. 2 evidences that TGF-b has a inhibition effect on the cytotoxicity of NK cells, even in the presence of IL-2 or IL-15, but the combination of IL-6 and IL-15 can restore the cytotoxicity of NK cells.

The Examples and Figures discussed above thus provide solid evidence that the combination of IL-6 plasmid and IL-15 plasmid can reduce tumor size by enhancing cytotoxicity of NK cells, even when TGF-β is present. Applicants therefore reiterate their position that the description of the invention present in the specification clearly enables the subject matter recited in the proposed new claims submitted herewith as required under 35 U.S.C. §112, First Paragraph.

Still further, with regard to the Examiner's statement on p. 6 of the Office Action that, "it is difficult to assess the nature of the binding interactions between the host immune cells and the xenogeneic tumor cells.", there is a great deal of evidence to the effect that NK cells kill xenogeneic target cells, such as the fact that human NK cells kill mouse target cells which are originally from mice (Bryceson et al., BLOOD (2006), 107, attached as **Exhibit C**: 159-166; Sivori, et al., PNAS (2002) 99: 4526-4531, attached as **Exhibit D**), dog NK cells kill human targets (Tan, et al., Journal of Immunology (1993) 1: 812-820, attached as **Exhibit E**), and bovine NK cells kill murine and human target cells (Storset, et al., Eur. J. Immunol. (2004) 34: 669-676, attached as **Exhibit F**).

Moreover, Example 4 demonstrates that CTVT can grow and cause mice to die xenogeneically in the group treated with Mock plasmid (see FIG. 8), and that the combined usage of plasmids comprising DNA sequences encoding human IL-6 and IL-15 can enhance the cytotoxicity of mice NK cells to lyse canine tumor cells, i.e., CTVT. Example 4 suggests that the plasmids encoding human IL-6 and IL-15 can enhance the cytotoxicity of NK cells xenogeneically, and that the NK cells of mice can lyse the xenogeneic tumor cells, i.e., CTVT cells. The results set forth in example 4 demonstrate that the effect of administering the combination of plasmids encoding human IL-6 and IL-15 on tumor reduction is independent of the genera . Since the tumor reduction strategy of the invention can work xenogeneically, it is reasonable to presume that the same strategy can be applied to a homogenetic model. For example, it is presumable that the combined usage of plasmids comprising DNA sequences encoding human IL-6 and IL-15 can enhance cytotoxicity of human NK cells to lyse human tumor cells.

Turning next to the Examiner's statement on p. 6 of the Office Action that, "Macrophages are known to target CTVT cells during tumor regression.....Marcrophages are known in the art to be activated by IL-15.", applicants note that in comparing the results achieved with the

group treated with anti-asialo GM1 antibody and plasmids encoding human IL-6 and IL-15 with those from the group treated with just plasmids encoding IL-6 and IL-15, Example 4, FIG. 6 and FIG. 8 of their specification reveal that when the function of NK cells is blocked by anti-asialo GM1 antibody, the tumor size can not be reduced even with treatment with plasmids encoding human IL-6 and IL-15. Those example and Figures thus provide evidence that the effect of combined usage of plasmids encoding human IL-6 and IL-15 on tumor reduction results from the cytotoxicity of NK cells, not from microphages activated by IL-15.

Furthermore, a series of supplemental experiments undertaken by the applicants further demonstrated that, in samples rendered devoid of macrophages by adhering the peripheral blood mononuclear cells on the cultured plates for 2 hours, the combination of IL-6 and IL-5 has an effect similar to that described above on TGF-ß and NK activity. The results of these tests are graphically represented in Figure 1 and Figure 2 attached as **Exhibit G** and **Exhibit H**, respectively to this Amendment.

As the discussion above demonstrates, therefore, applicants' proposed new claims 17-27 are completely enabled by the teachings contained in the application at the time of its filing. The Examiner is thus respectfully requested to reconsider and withdraw the rejection under 35 U.S.C. §112, First Paragraph.

Rejection Under 35 U.S.C. §112, Second Paragraph

Claim 4 is rejected under 35 U.S.C. §112, Second Paragraph (see, e.g., pps. 9-10 of the Office Action). The Examiner alleges that the claim is incomprehensible and requires that an appropriate correction be made.

In response to this ground for rejection, applicants respectfully submit that the cancellation of claim 4 in the present Response renders the rejection moot. Therefore, the Examiner is requested to reconsider and withdraw the rejection.

SUMMARY

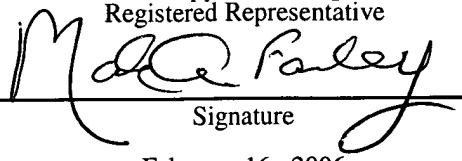
Applicants submit that the new claims and the remarks presented herein are believed to be sufficient to overcome all of the grounds for objection and/or rejection set forth in the Office Action and it is, therefore, respectfully requested that the Examiner reconsider and withdraw each of these objections and rejections. The application is, therefore, believed to be in condition for allowance, early notice of which would be appreciated.

If the Examiner believes that an Interview would advance the progress of this application, he is respectfully invited to telephone applicants' representative at the number below to arrange for such an interview.

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on: February 16, 2006

Mark A. Farley

Name of applicant, assignee or Registered Representative

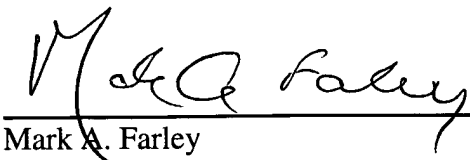


Signature

February 16 , 2006

Date of Signature

Respectfully submitted,



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Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion

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Freshly isolated, resting natural killer (NK) cells are generally less lytic against target cells than in vitro interleukin 2 (IL-2)–activated NK cells. To investigate the basis for this difference, the contribution of several receptors to activation of human NK cells was examined. Target-cell lysis by IL-2–activated NK cells in a redirected, antibody-dependent cytotoxicity assay was triggered by a number of receptors. In contrast, cytotoxicity by resting NK cells was induced only by CD16, and not by NKp46, NKG2D, 2B4 (CD244), DNAM-1 (CD226), or CD2. Calcium flux in resting NK cells was induced with antibodies to CD16 and, to a weaker extent, antibodies to NKp46 and 2B4. Although NKp46 did not enhance CD16-mediated calcium flux, it synergized with all other receptors. 2B4 synergized with 3 other receptors, NKG2D and DNAM-1 each synergized with 2 other receptors, and CD2 synergized with NKp46 only. Resting NK cells were induced to secrete tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ), and to kill target cells by engagement of specific, pair-wise combinations of receptors. Therefore, natural cytotoxicity by resting NK cells is induced only by mutual costimulation of nonactivating receptors. These results reveal distinct and specific patterns of synergy among receptors on resting NK cells. (Blood. 2006; 107:159-166)

Introduction

Natural killer (NK) cells are characterized by cytolytic activity against susceptible target cells and by the secretion of cytokines, such as tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ). NK cells discriminate between normal and abnormal cells (infected or transformed) through engagement and dynamic integration of multiple signaling pathways, which are initiated by germline-encoded receptors.[1-3] Healthy cells are protected from NK-cell–mediated lysis by expression of major histocompatibility complex (MHC) class I ligands for NK-cell–inhibitory receptors.[1,4] However, de novo expression of ligands for NK-cell activation receptor NKG2D can trigger natural cytotoxicity against MHC class I^+ target cells.[5,6] A number of structurally distinct receptors have been implicated in activation of NK-cell effector functions. It is not yet clear if any one receptor is necessary or sufficient to activate NK cells and to what extent activation receptors may be redundant. Activation receptors can be grouped in 3 categories: receptors that signal through immunoreceptor tyrosine-based activation motif (ITAM)–containing subunits (eg CD16, NKp46, NKp44), the DAP10-associated receptor NKG2D, and several other receptors (eg CD2, 2B4, DNAM-1) that signal by different pathways.

CD16 (FcγRIII), a low-affinity receptor for IgG, is associated with the ITAM-containing FcεRI γ chain and T-cell receptor (TCR) ζ chain. NKp46 and NKp30 are associated with the TCR ζ chain.[7] NKp44, KIR2DS, and CD94/NKG2C are associated with the ITAM-containing DAP12. Natural cytotoxicity receptors (NCRs), which include NKp46, NKp44, and NKp30, play a major role in NK-cell cytotoxicity against transformed cells.[8] Although ligands of NCRs have not been identified, antibodies against NCRs have been used to block lysis of tumor cells by interleukin 2 (IL-2)–activated and resting NK cells.[9-11] However, in the mouse, Syk/ZAP70-independent natural cytotoxicity by NK cells was observed, implying that natural cytotoxicity can occur independently of ITAM-based activation signals.[12,13]

NKG2D can signal through both DAP10 and DAP12 in mice,[14,15] whereas human NKG2D associates only with DAP10.[16-18] DAP10 is a signaling subunit that carries a phosphatidylinositol-3 kinase-binding motif.[16] Ligands for NKG2D, such as MICA and ULBP, are expressed on some tumor cells, and on infected or stressed cells.[19] Experiments have suggested that NKG2D signals are sufficient to activate NK-cell functions.[20-22] Lysis of certain tumor cells by resting NK cells and by IL-2–activated NK cells can be blocked by antibodies to NKG2D.[11,23] The importance of ligands for NKG2D in immune defense is underscored by strategies developed by viruses to interfere with their expression.[19,24,25]

Several other receptors activate NK cells by signaling through their own cytoplasmic tail. 2B4 (CD244) recruits SAP and Fyn through cytoplasmic tyrosine-based motifs.[26,27] The ligand of 2B4 is CD48, which is expressed on hematopoietic cells.[28] CD2 and NKp80 signal through largely unknown pathways. CD2 binds to LFA-3 (CD58).[29] The ligand of NKp80 is unknown. DNAM-1 is associated with LFA-1 in NK cells,[30] is phosphorylated by a PKC,[31]

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and binds to CD155 and CD112.[32] Antibodies to DNAM-1 inhibit NK-cell cytotoxicity toward tumor cells.[32-34]

Anti-CD16 mouse hybridomas are lysed by human NK cells in so-called redirected lysis assays, in which CD16 is cross-linked by surface IgG.[35,36] Likewise, lysis of mouse FcR$^+$ P815 cells, in a redirected, antibody-dependent lysis assays, by IL-2–activated human NK cells can be induced by independent engagement of CD16,[36,37] NKp46,[38] NKp44,[39] NKp30,[10] NKp80,[40] NKG2D,[11,41] 2B4,[42] CD2,[43] DNAM-1,[31] KIR2DS,[44] CD94/NKG2C,[45] and KIR2DL4,[46,47] suggesting extensive redundancy in activation pathways. Most published studies have used polyclonal or clonal NK cells that have been expanded in IL-2. Because resting NK cells cannot be maintained very long in the absence of cytokines, much less is known about their requirements for activation of cytotoxicity and cytokine secretion. Here, we tested activation of resting NK cells by several receptors and by pair-wise combinations of receptors. For most NK-cell receptors, it is still unknown if they are capable of triggering NK-cell effector function independently or if they can only serve as costimulating receptors. Apart from the FcR CD16, which was sufficient for activation of cytotoxicity and cytokine release by resting NK cells, all NK-cell receptors tested required coengagement of another receptor for activation. Clear synergies between specific pairs of coactivating receptors were observed in the activation of cytotoxicity and cytokine secretion by resting NK cells.

Material and methods

Cells

Human NK-cell populations were isolated from peripheral blood by negative selection using an NK isolation kit (Miltenyi Biotec, Auburn, CA). Polyclonal IL-2–activated NK cells were expanded in Iscove modified Dulbecco medium (IMDM; Invitrogen, Carlsbad, CA) supplemented with 10% human serum (Valley Biomedical, Winchester, VA), 100 U/mL recombinant IL-2 (rIL-2; Hoffmann-La Roche, Basel, Switzerland), and 10% purified human IL-2 (Hemagen, Columbia, MD). Resting NK cells were resuspended in the same medium without IL-2 and were used within 1 to 4 days after isolation. These cells were 95% to 99% CD3$^-$CD56$^+$ as determined by flow cytometry. Prior to experiments, trypan blue exclusion was used to assess cell viability. After 4 days in culture, viability of resting NK cells was typically more than 90%. Cell-surface expression of activating receptors and the relative distribution of CD56^{dim} and CD56^{bright} NK cells did not change significantly during the culture. The mouse mastocytoma cell line P815 and the human erythroleukemia cell line K562 (both from American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS; all from Invitrogen).

Antibodies

For flow cytometric analysis, antibody-dependent redirected lysis, bead stimulation, and calcium flux measurements, the following monoclonal antibodies (mAbs; all mouse IgG1 isotype) were used: anti-CD2 (clone RPA-2.10), anti-CD3 (clone UCHT1), anti-CD16 (clone 3G8), anti-CD56 (clone B159), anti-CD107a (clone H4A3), anti-CD226/DNAM-1 (clone DX11), anti-NKG2D (clone 1D11), and isotype control (clone MOPC-21) from BD Biosciences (Franklin Lakes, NJ). Anti-CD244/2B4 (clone C1.7) and anti-NKp46 (clone BAB281) were from Beckman Coulter (Fullerton, CA). Anti-NKG2D (clone 149810) was from R&D Systems (Minneapolis, MN). Isotype control mouse IgG1 (clone MOPC-21) was from Sigma (St Louis, MO). Secondary goat F(ab')$_2$ anti–mouse IgG was from Jackson ImmunoResearch (West Grove, PA).

^{51}Cr-release assay

A standard 3-hour ^{51}Cr-release assay was performed as described.[48] Briefly, P815 or K562 target cells were labeled with 0.5 mCi/mL (19 MBq/mL) ^{51}Cr (Amersham Pharmacia, Piscataway, NJ) in saline solution for 1 hour at 37°C. Cells were washed 3 times in phosphate-buffered saline (PBS) and resuspended at 1×10^6 cells/mL in IMDM supplemented with 10% FBS. P815 cells were incubated for 30 minutes at room temperature with 10 μg/mL mAb. Cells were spun down, resuspended in medium, and plated at 1×10^4 cells/well in triplicate. NK cells were washed, resuspended in IMDM supplemented with 10% FBS, and added to wells at the indicated effector-to-target (E/T) cell ratios. The supernatants were measured for ^{51}Cr release on a γ-counter (Cobra II Auto-Gamma, Global Medical Instruments, Ramsey, MN).

Flow cytometric and single-cell Ca^{2+} flux analysis

NK cells were stained with mAbs at 10 μg/mL in 50 μL Hanks buffered salt solution (HBSS) with Ca^{2+} and Mg^{2+} (Biosource, Camarillo, CA) supplemented with 1% FBS for 30 minutes on ice. Then 300 μL dye-loading buffer (HBSS 1% FBS, 2 μM Fluo-4 AM, 5 μM Fura Red AM [both Molecular Probes, Eugene, OR] and 5 mM probenecid σ) were added and cells were further incubated for 30 minutes on ice. Cells were pelleted by centrifugation and washed once in HBSS 1% FBS. Cells were kept on ice and resuspended in 300 μL HBSS 1% FBS just prior to a 5-minute incubation in a waterbath at 37°C. Samples were vortexed and analyzed by flow cytometry (FACS Calibur, BD Bioscience). After 30 seconds, tubes were briefly removed, 4 μg goat anti–mouse F(ab')$_2$ antibodies were added, samples were vortexed, placed back on the flow cytometer, and events were acquired for 4 minutes. Data were analyzed with FlowJo software (Treestar, Ashland, OR). NK cells were gated on forward scatter/side scatter plots. The ratio between the mean fluorescence intensity of FL-1 and FL-3 was calculated and plotted as a function of time. The baseline for isotype control-stimulated cells was set to 1, and this factor was used to normalize the other plots in every batch.

For microscopic single-cell analysis, resting NK cells were resuspended in culture medium containing 0.4 μM Fluo-4 AM, 4 μM Fura Red AM, and 5 mM probenecid at 2×10^6 cells/mL and incubated at room temperature for 30 minutes. The cells were washed once in HBSS 1% FBS and resuspended at 2×10^6 cells/mL in HBSS 1% FBS. Aliquots of 0.5 mL were added to poly-L-lysine–coated (Sigma) wells of Lab-Tek II chambered coverglasses (Nunc, Roskilde, Denmark). The cells were allowed to settle onto the coverglass for 15 minutes at room temperature. The medium was aspirated from the chambers and replaced with 300 μL cold HBSS 1% FBS containing 3 μg of each of the appropriate mAbs. The chambers were incubated for 30 minutes on ice. Cells were washed once with 1 mL cold HBSS 1% FBS, and 400 μL cold HBSS 1% FBS was placed in each chamber. Prior to acquisition on the confocal microscope, each chamber was warmed in a 37°C, 5% CO_2 incubator for 5 minutes. Data were acquired on a Zeiss LSM510 confocal microscope using a × 40 oil-immersion lens (Zeiss, Göttingen, Germany). The microscope was set in λ scan mode so that wavelengths from 499 to 670 nm were acquired in a single pass. The pinhole was adjusted so that optic slices of approximately 2 μm were acquired. Scan speed was set so that one scan was completed every 4 seconds. Thirty seconds after the beginning of acquisition, secondary cross-linking goat anti–mouse F(ab')$_2$ antibody was added to a final concentration of 13 μg/mL. Scanning was continued for 10 minutes. After acquisition was complete, the data were processed by linear unmixing, using control spectra acquired from cells loaded individually with either Fluo-4 or Fura-Red. After unmixing, individual cells were analyzed to give the fluorescence values of the Fluo-4 and Fura Red for the entire time series, which were used to calculate the ratio of the Fluo-4 fluorescence to the Fura Red fluorescence.

CD107a degranulation assay

P815 cells (2×10^5) were incubated for 15 minutes at room temperature with 5 μg/mL mAbs. Cells were spun down and resuspended with 1×10^5 resting NK cells in IMDM supplemented with 10% FBS. Cells were spun

down for 3 minutes at 17*g* and incubated for 2 hours at 37°C. Thereafter, cells were spun down and cell pellets were resuspended in PBS 2% FBS and stained with PE-conjugated anti-CD56 and FITC-conjugated anti-CD107a mAbs, followed by flow cytometric analysis.

Bead stimulation and ELISA

Beads with a diameter of 4.5 μm were used to stimulate NK cells at a ratio of 8 beads per cell. To coat beads with saturating amounts of mAb, 4×10^7 goat anti-mouse-coated beads (Dynabeads M-450; Dynal, Oslo, Norway) were incubated with 3 μg premixed mAbs in PBS 2% FBS for 1 hour at 4°C. Beads were washed 3 times in PBS 2% FBS to remove excess mAbs. For cell stimulation, 4×10^6 beads were incubated with 5×10^5 resting NK cells in 500 μL IMDM supplemented with 10% human serum for 2 or 6 hours at 37°C. The cultures were rotated end-over-end during the stimulation. Supernatants were stored at −20°C and analyzed with TNF-α enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) or IFN-γ ELISA kit (Pierce, Rockford, IL).

Results

Induction of cytotoxicity by receptors on resting NK cells

Cytotoxicity by IL-2–activated NK cells and by resting NK cells was compared in redirected, lysis assays. The mouse FcR^+ cell line P815 was incubated with mAbs to several NK-cell receptors and mixed with NK cells. ^{51}Cr release by P815 cells at several E/T cell ratios was measured after 3 hours (Figure 1). In several independent experiments, up to 20% lysis was observed when P815 cells were incubated with IL-2–activated NK cells at an E/T cell ratio of 10 (not shown). This lysis was not enhanced by the presence of isotype control IgG1 (11% ± 8.0% [mean ± SD] specific lysis at an E/T ratio of 10:1, 5 independent experiments), and of mAbs to CD56 or CD2 (Figure 1A). The mAbs to CD16, NKp46, NKG2D, 2B4, and DNAM-1 each augmented lysis of P815 target cells in the presence of IL-2–activated NK cells (Figure 1A). Notably, at lower E/T cell ratios, CD16 was the strongest inducer of cytotoxicity by IL-2–activated NK cells (Figure 1A).

Freshly isolated, resting NK cells did not induce lysis of P815 cells either in the absence (not shown) or in the presence of isotype control IgG1 (1.0% ± 1.8% specific lysis at an E/T cell ratio of 10:1; n = 7; Figure 1B). A mAb to CD16 induced 35% to 65% lysis of P815 cells at an E/T cell ratio of 10 (51% ± 13% specific lysis; n = 7). The mAbs to NKp46, NKG2D, 2B4, CD2, DNAM-1, or CD56 induced little or no lysis of P815 target cells (Figure 1B). With freshly isolated NK cells from some donors, up to 10% lysis with mAb to 2B4 (6.9% ± 4.7% specific lysis at an E/T cell ratio of 10:1, n = 7) or DNAM-1 (5.2% ± 4.9% specific lysis at an E/T cell ratio of 10:1; n = 6) was observed. Less lysis was observed with a mAb to NKp46 (2.0% ± 1.7% specific lysis at an E/T cell ratio of 10:1, n = 6). No lysis was observed with NK cells from any donor with mAbs to NKG2D, CD2, and CD56 (Figure 1B).

In some instances, natural ligands of NKG2D activated NK cells more efficiently than mAbs to NKG2D.[49] We therefore investigated whether incubation of P815 target cells with MICA-Fc or ULBP1-Fc could induce lysis by resting NK cells. MICA-Fc and ULBP1-Fc did not induce lysis by resting NK cells, even though each one augmented the lysis of P815 cells by IL-2–activated NK cells to the same extent as NKG2D mAb (not shown).

The lack of cytotoxicity by receptor engagement on resting NK cells could be explained in different ways. Resting NK cells may be intrinsically less lytic or much stronger signals may be required to induce lysis by resting NK cells. However, resting NK cells readily lysed K562 cells to a similar degree as IL-2–activated NK cells (Figure 1C), indicating that resting NK cells are fully capable of natural cytotoxicity, if provided with sufficient activation signals. Intracellular staining revealed high expression of perforin in both resting and IL-2–activated NK cells (data not shown). A comparison of expression of activating receptors on resting and IL-2–activated NK cells revealed that resting NK cells express high levels of the receptors studied here (Figure 2). Of note, IL-2–activated NK cells expressed more uniformly high levels of CD2 and CD56, as compared to resting NK cells (Figure 2). The small increase in receptor expression on IL-2–activated NK cells (eg, NKp46 and NKG2D) is unlikely to explain the observed differences in cytotoxicity by resting and IL-2–activated NK cells. Additional explanations include that some receptors may not signal in resting NK cells or that resting NK cells may have good cytolytic potential but require additional signals to induce cytotoxicity.

Synergistic activation of intracellular Ca^{2+} mobilization in resting NK cells by pair-wise combinations of receptors

Intracellular Ca^{2+} concentration was measured in resting NK cells after cross-linking different receptors. NKp46, 2B4, NKG2D, and DNAM-1 are expressed on all NK cells. CD16 is expressed on NK cells of the $CD56^{dim}$ subset, which represented more than 90% of the total NK cell population. Therefore, analysis of activation by these receptors was performed on a forward scatter/side scatter gate for total NK cells. Because CD2 is expressed on a subset of resting NK cells, cells were stained with PE-conjugated anti-CD2 and gated on $CD2^{bright}$ cells. To investigate the relative potency of individual receptors and the effect of co-crosslinking different receptor combinations, time-course analysis was performed with resting NK cells preincubated with mAbs to a single receptor or to pair-wise combinations of receptors. Cells were loaded with the Ca^{2+} sensitive dyes Fluo-4 and Fura Red and analyzed by flow cytometry (Figure 3). After acquisition of baseline fluorescence, secondary goat $F(ab')_2$ antimouse fragments were added to cross-link receptors. The FL-1/FL-3 ratio was recorded over 4 minutes (Figure 3A). The mean peak FL-1/FL-3 value from several independent experiments was also calculated (Figure 3B). The mAb to CD16 elevated baseline Ca^{2+} without cross-linking, and

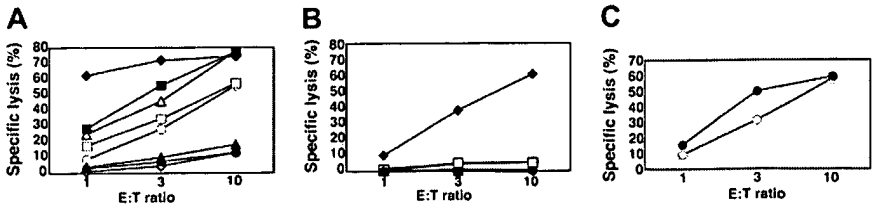

Figure 1. Engagement of CD16, but not NK-cell receptors NKp46, NKG2D, 2B4, DNAM-1, or CD2, induces cytotoxicity by resting NK cells. Redirected lysis assay of P815 target cells with IL-2–activated NK cells (A) or resting NK cells (B) at indicated E/T cell ratios. P815 cells were preincubated with IgG1 mAbs to specific NK-cell receptors, as indicated. ◇ indicates isotype control antibody; ◆, anti-CD16; □, anti-NKp46; ■, anti-NKG2D; △, anti-2B4; ▲, anti-CD2; ○, anti–DNAM-1; and ●, anti-CD56. (C) Lysis of K562 cells by resting (○) and IL-2–activated (●) NK cells. Experiments are representative of at least 5 independent experiments.

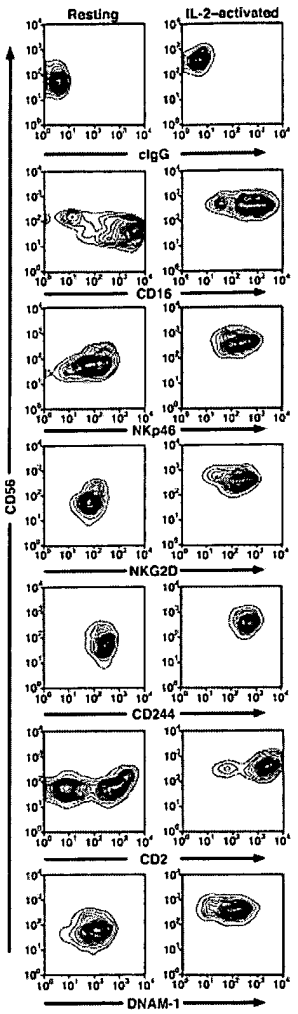

Figure 2. Expression of activating receptors on resting and IL-2–activated NK cells. Freshly isolated, resting NK cells or long-term IL-2–cultured NK cells were stained with directly conjugated mAbs to the indicated receptors (x-axis) and anti-CD56 (y-axis).

this was further augmented by cross-linking with secondary antibodies (Figure 3A). Cross-linking of NKp46 and of 2B4 also led to rises in intracellular Ca^{2+}, but the peak FL-1/FL-3 ratios were significantly lower and the kinetics were slower than responses elicited by CD16 cross-linking (Figure 3). Cross-linking of mAb to NKG2D or DNAM-1 induced only a very small but reproducible rise in intracellular Ca^{2+}. Cross-linking CD2 produced only minor elevations in intracellular Ca^{2+} (Figure 3) and cross-linking of CD56 did not produce any rises in Ca^{2+} (Figure 3B and data not shown).

Co-crosslinking CD16 with NKG2D, 2B4, CD2, or DNAM-1 enhanced the peak Ca^{2+} (Figure 3). Co–cross-linking with the other ITAM-containing receptor NKp46 (Figure 3) or with CD56 (not shown) did not enhance Ca^{2+} flux. Although NKp46 mAb induced modest elevations in Ca^{2+} by itself, co-crosslinking with mAbs to NKG2D, 2B4, CD2, or DNAM-1 significantly increased the rise in intracellular Ca^{2+} (Figure 3). 2B4 induced significant elevations in intracellular Ca^{2+} in combination with CD16, NKp46, NKG2D, or DNAM-1, but not with CD2 or CD56 (Figure 3 and data not shown). NKG2D cross-linking synergized with CD16, NKp46, or 2B4. A small enhancement was also observed when NKG2D and DNAM-1 were co-crosslinked, but no synergy was observed on cross-linking with CD2 (Figure 3). Cross-linking CD2 and DNAM-1 did not produce any significant rise in intracellular Ca^{2+} (Figure 3). These results revealed a complex pattern of synergy among receptors in activation of resting NK cells.

Flow cytometry cannot follow the kinetics of Ca^{2+} regulation in individual cells. Recording of Ca^{2+} fluctuations in individual T cells revealed important differences in functional outcome of oscillating versus sustained Ca^{2+} fluxes.[50] We performed microscopy experiments to follow intracellular Ca^{2+} responses elicited in individual NK cells after receptor cross-linking or co-crosslinking (Figure 4). Resting NK cells were placed on poly-L-lysine–coated coverslips, incubated with mAbs, and loaded with Ca^{2+}-sensitive dyes Fluo-4 and Fura Red. Recordings of individual cells revealed that CD16 cross-linking elicited varied responses (Figure 4A), in comparison to baseline Ca^{2+} fluctuations observed with isotype control mAb-treated cells (Figure 4B). In some cells, CD16 cross-linking induced a sharp but transient rise in intracellular Ca^{2+}. In other cells, lower peaks but more oscillation was observed. Whereas NKG2D cross-linking produced little or no rise in baseline intracellular Ca^{2+} (Figure 4C), cross-linking of CD16 and NKG2D resulted in a more sustained elevation in Ca^{2+} in most cells (Figure 4D). Cross-linking of 2B4 induced sustained rises in intracellular Ca^{2+} (Figure 4E), and these were further enhanced by co-crosslinking with NKG2D (Figure 4F). Overall, data match those obtained by flow cytometry.

In conclusion, each receptor is capable of contributing a signal in resting NK cells, which results in Ca^{2+} mobilization when costimulated by another receptor. Some receptors can elicit Ca^{2+} flux directly, in the absence of adhesion or coengagement of other receptors.

Synergistic Ca^{2+} flux correlates with degranulation

Phospholipase C-γ activation and Ca^{2+} mobilization are among the early steps leading to perforin-dependent NK-cell cytotoxicity.[51] Exocytosis of secretory lysosomes, which are lytic granules, is required to complete this process. An assay was used to measure degranulation based on cell-surface expression of the lysosomal protein CD107a (LAMP-1).[52] Resting NK cells did not stain with anti-CD107a mAb, relative to isotype control mAb staining, and incubation with P815 cells and isotype control antibody did not induce CD107a surface expression (Figure 5). When NK cells were incubated with anti-CD16 mAb and FcR^+ P815 cells for cross-linking, CD107a expression was detected on 50% of the NK cells (52% ± 9.2% [mean ± SD] of $CD107a^+CD56^+$ NK cells, 7 independent experiments; Figure 5). Notably, degranulation was observed on the $CD56^{dim}CD16^+$ but not the $CD56^{bright}CD16^-$ NK-cell subset (Figure 5). Alone, anti-NKG2D and anti-2B4 mAbs did not induce surface expression of CD107a (1.3% ± 0.6% and 2.7% ± 1.3%, respectively, 7 independent experiments; Figure 5). Therefore, CD107a surface expression correlated with the observed cytolytic activity by resting NK cells (Figure 1B). When resting NK cells were incubated with a combination of anti-CD16 and anti-NKG2D mAbs, CD107a surface expression increased somewhat relative to that induced by anti-CD16 mAb alone (56% ± 8.3%, 7 independent experiments; Figure 5). Interestingly, the combination of anti-NKG2D and anti-2B4 mAbs induced a synergistic increase in CD107a surface expression (13% ± 6.1%, 7 independent experiments; Figure 5), correlating with the observed synergy for Ca^{2+} flux.

Stimulation of TNF-α and IFN-γ secretion

To evaluate which receptor or receptor combination could induce cytokine secretion, resting NK cells were incubated for 2

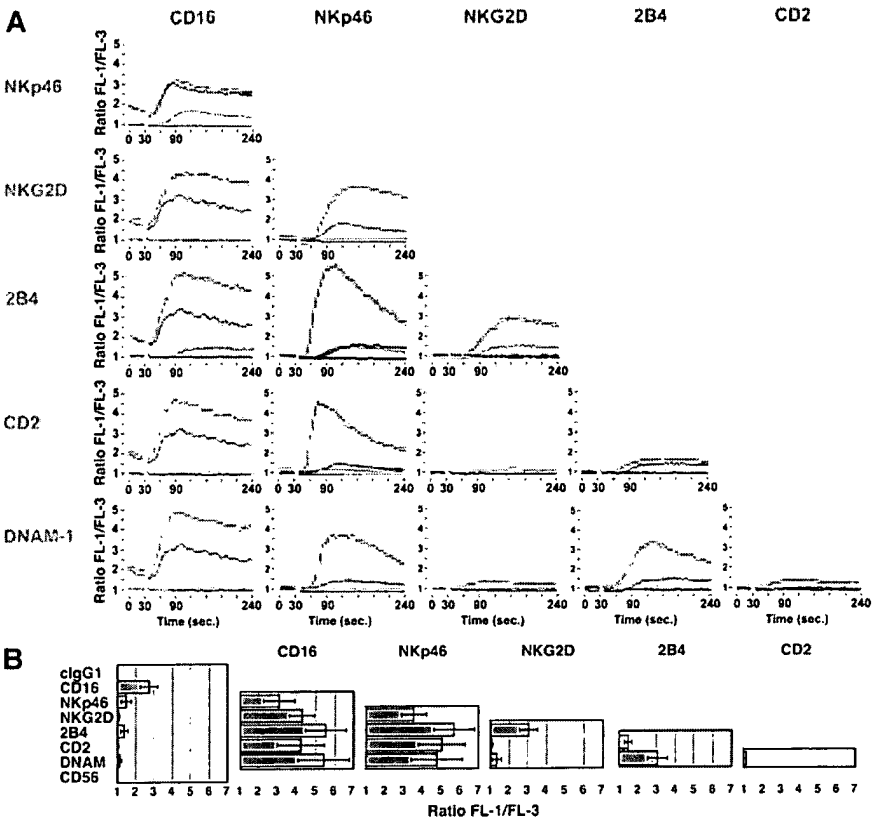

Figure 3. Synergistic activation of Ca^{2+} flux in resting NK cells by co-crosslinking pair-wise combinations of receptors. (A-B) NK cells were preincubated with mAbs to indicated receptors on ice, loaded with Fluo-4 and Fura Red, resuspended in HBSS 1% FBS, and prewarmed at 37°C. Cells were analyzed by flow cytometry. After 30 seconds, secondary $F(ab')_2$ goat anti–mouse IgG was added to each sample. (A) FL-1/FL-3 ratios are plotted as a function of time. Black lines show activation with isotype control. Blue lines show activation by the single receptors, indicated in blue. Green lines show activation by the single receptors, indicated in green. Red lines show activation by the combination of both receptors. (B) The peak FL-1/FL-3 ratio after crosslinking of indicated receptor combinations was measured in several independent experiments. Bars indicate the SD (≥ 3 independent experiments).

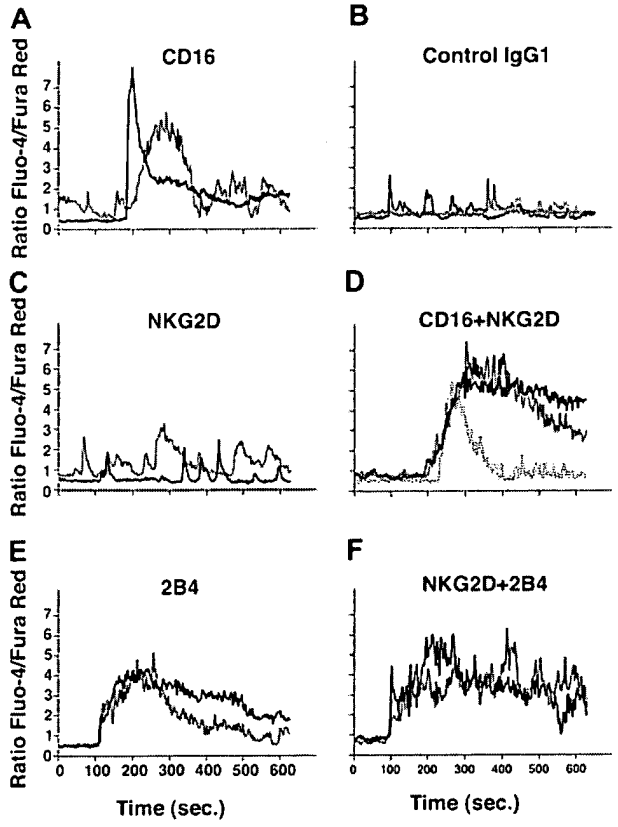

Figure 4. Single-cell Ca^{2+} flux analysis. (A-F) Resting NK cells were loaded with Fluo-4 and Fura Red, placed on coverslips, and stained with antibodies as indicated. Cells were washed, resuspended in HBSS 1% FBS, and prewarmed at 37°C. Fluorescence was measured by confocal microscopy. Thirty seconds after the beginning of each scan, secondary $F(ab')_2$ goat anti–mouse IgG was added to each sample. Traces of the Fluo-4/Fura Red ratio of 2 or 3 representative NK cells are shown.

hours at 37°C with beads coated with mAbs to NK-cell receptors. TNF-α secretion was quantified by ELISA. The mAb to CD16 was sufficient to induce TNF-α secretion by resting NK cells (Figure 6A). By comparison, mAbs to other receptors did not induce TNF-α secretion (Figure 6A). Therefore, the difference between CD16 and the other receptors was even more striking than it had been in Ca^{2+} flux measurements. All pair-wise combinations of mAbs were tested for induction of TNF-α secretion (Figure 6A). In these experiments, mAbs to 2 receptors were added together on the same beads. NKp46 synergized with NKG2D, 2B4, and CD2, and to a lesser extent

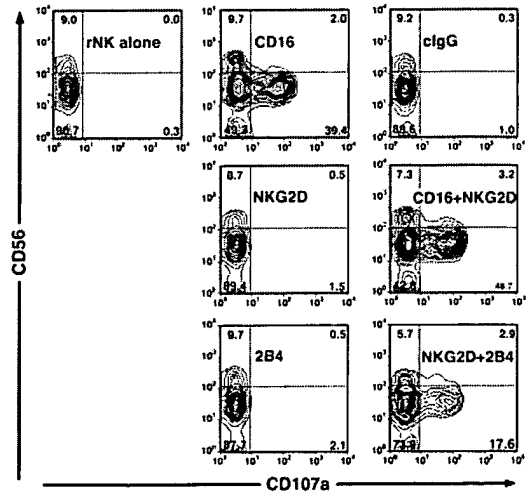

Figure 5. Degranulation by resting NK cells induced by NKG2D and 2B4 synergism. NK cells were incubated for 2 hours either alone or with P815 cells and mAbs as specified. Cells were stained with anti-CD56 and isotype control or anti-CD107a mAbs. Plots were gated on a forward scatter/side scatter lymphocyte gate. The experiment is representative of 7 independent experiments.

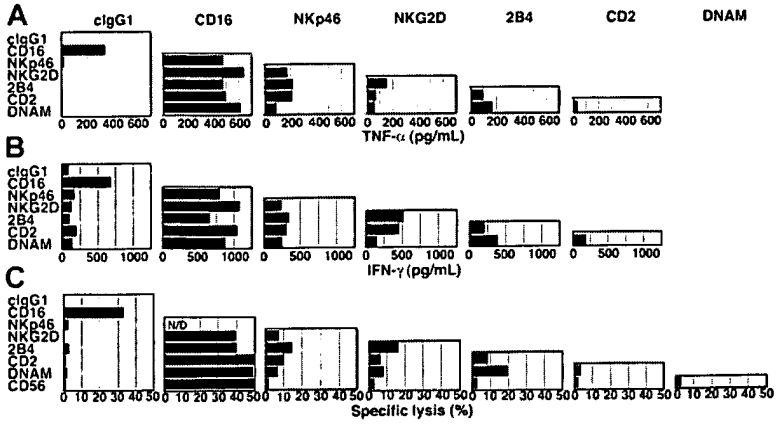

Figure 6. TNF-α secretion, IFN-γ secretion, and cytotoxicity induced by pair-wise combinations of NK-cell receptors. TNF-α (A) and IFN-γ (B) secretion by resting NK cells after 2 or 6 hours of stimulation, respectively, with beads coated with specific combinations of mAbs, as indicated. (C) Redirected lysis of P815 cells by resting NK cells at an E/T cell ratio of 10, for 3 hours at 37°C. P815 target cells were preincubated with combinations of mAbs to specific NK-cell receptors, as indicated.

with DNAM-1 (Figure 6A). In addition, clear synergy was observed between NKG2D and 2B4, 2B4 with DNAM-1, and 2B4 with CD2 (Figure 6A). Weaker stimulations were obtained with combinations of NKG2D, CD2, and DNAM-1 (Figure 6A). Therefore, synergies similar to those observed for the induction of Ca^{2+} flux occurred for the induction of TNF-α secretion. Secretion of IFN-γ was also measured after a 6-hour stimulation (Figure 6B). In repeated experiments, secretion of IFN-γ responded to the same combinations of receptor signals as TNF-α.

Cytotoxicity induced by pair-wise combinations of receptors

To test whether combinations of receptors that synergize to activate Ca^{2+} flux and cytokine secretion could also signal for cytotoxicity by resting NK cells, the redirected lysis assay shown in Figure 1 was used. All pair-wise combinations of receptors were tested for lysis at an E/T cell ratio of 10 (Figure 6C). The strongest synergies were observed with 2B4 in combination with NKp46, NKG2D, and DNAM-1. In addition, as seen with induction of Ca^{2+} flux and cytokine secretion, receptor NKp46 synergized with all other receptors. Overall, the pattern of synergies (Figure 7) was similar in all assays used. We conclude that resting NK cells are quite capable of killing target cells, given the right combination of signals. Furthermore, of all the receptors tested, each one is capable of contributing a signal in resting NK cells.

Discussion

The data presented here show that responses by resting NK cells, in the absence of exogenous cytokines, can be induced by combinations of activation receptors. Responses measured were Ca^{2+} flux triggered by cross-linked mAbs in solution, TNF-α and IFN-γ secretion induced by mAbs attached to beads, and cytotoxicity against an FcR^+ target cell in the presence of mAbs directed to NK-cell activation receptors. Except for the FcR CD16, which mediates antibody-dependent cellular cytotoxicity (ADCC) and not natural cytotoxicity, no single receptor tested—NKp46, NKG2D, 2B4, DNAM-1, or CD2—was sufficient to activate considerable cytotoxicity or cytokine secretion. However, each one of these receptors contributes a signal in resting NK cells, as shown by synergistic activation by specific pair-wise combinations of receptors. Resting NK cells are not inherently less responsive than IL-2–activated NK cells, but the regulation of their activation is more stringent.

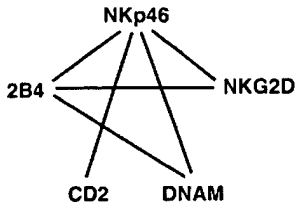

Figure 7. Schematic representation of synergies among receptors in resting NK cells. Solid lines represent strong synergies between pairs of receptors.

A hierarchy among receptors for the activation of resting NK cells was revealed. CD16 was unique in its ability to induce cytokine secretion by resting NK cells without additional signals. With regard to cytotoxicity, it is possible that recognition of mouse ligands on P815 cells by human NK cells contributes to activation. P815 cells express mouse ICAM-1, which is a ligand for human LFA-1.[53] Blocking antibodies to LFA-1 reduced the CD16-mediated cytotoxicity toward target cells.[35] In Ca^{2+} flux experiments, activation by CD16 was enhanced by all other receptors, except NKp46, suggesting that ITAM-based signals do not enhance one another. The results imply a difference in signaling via the ITAM chains associated with CD16 and those associated with NKp46. The mAb to NKp46, in the absence of further cross-linking, was not sufficient to mobilize Ca^{2+} in resting NK cells. Further, cross-linking of NKp46 resulted in lower Ca^{2+} flux as compared to CD16. CD16 associates with ITAM-containing chain (FcεRI γ and TCR ζ) complexes through a negatively charged aspartate residue in the transmembrane, whereas NKp46 has a positively charged arginine residue in the transmembrane. Cross-linking of NKp46 on resting NK cells was insufficient for full activation, but if provided with additional signals, NKp46 can potentiate activation. NKp46 ranks high in the hierarchy, but below CD16, because it synergizes with all 4 other receptors. The observed synergies among receptors involved in natural cytotoxicity are depicted in Figure 7.

2B4 synergizes with the 3 receptors, NKp46, NKG2D, and DNAM-1. NKG2D and DNAM-1 each synergize with the 2 receptors, NKp46 and 2B4. Finally, CD2 is unique in its exclusive synergy with ITAM-associated receptors. Although the signaling pathways for most of these receptors are still poorly understood, evidence indicates that each receptor signals by different pathways.[54,55] The NKG2D/DAP10 complex activates phosphatidylinositol-3 kinase, phospholipase C-γ and Vav.[22] 2B4 recruits SAP and Fyn, through phosphorylated tyrosine motifs.[26,27] DNAM-1 is associated with LFA-1 in NK cells and is phosphorylated by PKC.[30,31] CD2 binds the adaptor protein CD2AP, as shown in T cells, and provides a link to Wiskott-Aldrich syndrome protein-mediated actin cytoskeleton remodeling.[56,57] The unique pattern of

receptor combinations that provide synergy is consistent with the use of different signaling modules by each receptor to induce activation. It will be necessary to identify signaling components for each of the receptors to determine the basis for the synergies.

Our data have revealed a complex hierarchy, synergy, and redundancy among activation receptors on NK cells. A hierarchy of CD16 > NKp46 > 2B4 > NKG2D, DNAM-1 > CD2 can be established on the basis of requirements for NK-cell activation. It should be noted that CD16 is not a natural cytotoxicity receptor. CD16 mediates ADCC, which leads to the elimination of antibody-coated target cells. Because different combinations of different signals can each result in activation of NK-cell function, including natural cytotoxicity, redundancy is apparent. For example, the combination of NKp46 and CD2 is sufficient to activate, but the combination of 2 other signals, such as DNAM-1 and 2B4, can also activate. It is likely that this kind of redundancy forms the basis for the natural cytotoxicity observed with NK cells from mice with a double Syk/ZAP70 deficiency, even on target cells that lacked ligands for NKG2D.[12] Several combinations of signals, excluding ITAM- and DAP10-based signals, are still available that can result in activation of cytotoxicity.

Activation of resting NK cells by synergistic signals raises the issue of terminology used to describe NK-cell receptors. NK cells do not have a dominant activation receptor, in the sense used to describe the TCR, except for the ADCC induced by CD16. NK cells use combinations of synergistic receptors to mediate natural cytotoxicity. The term "costimulation" could be confusing because it means something different in the context of T cells. "Coactivation" receptors may be a useful term to describe the NK-cell activation receptors studied here.

The importance of a tight regulation of natural cytotoxicity by inhibitory receptors on NK cells is obvious, considering that ligands for several of the coactivation receptors are widely expressed. LFA-3 (CD58), the ligand for CD2, is broadly expressed, whereas expression of CD48, a ligand for 2B4, is confined to hematopoietic cells and a subset of endothelial cells.[58] Ligands for DNAM-1, CD155 and CD112, are transcribed in a wide variety of tissues.[59,60] Ligands of NKp46 and of other NCRs have not been identified yet, but may include a carbohydrate component.[61-63] Cells that have up-regulated expression of ligands for NKG2D become sensitive to lysis by NK cells. Our data suggest that NKG2D does not signal alone but provides a coactivation signal to pre-existing signals from other receptors, such as 2B4 and NCRs. Thus, the induced expression of ligands for NK-cell receptors may suffice to override the balance between activation and inhibition. The distribution of ligands in vivo must determine degrees of sensitivity to NK cells. Some of the most widely expressed ligands are those for receptors DNAM-1, CD2, and NKG2D. Combinations of these receptors did not produce robust NK-cell activation. We propose that a more restricted expression of ligands for 2B4, NKp46, and other NK-cell receptors with similar coactivation potential could confine NK-cell alloreactivity to hematopoietic cells.

Acknowledgments

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Early expression of triggering receptors and regulatory role of 2B4 in human natural killer cell precursors undergoing *in vitro* differentiation

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In this study we analyzed the progression of cell surface receptor expression during the *in vitro*-induced human natural killer (NK) cell maturation from CD34$^+$ Lin$^-$ cell precursors. NKp46 and NKp30, two major triggering receptors that play a central role in natural cytotoxicity, were expressed before the HLA class I-specific inhibitory receptors. Moreover, their appearance at the cell surface correlated with the acquisition of cytolytic activity by developing NK cells. Although the early expression of triggering receptors may provide activating signals required for inducing further cell differentiation, it may also affect the self-tolerance of developing NK cells. Our data show that a fail-safe mechanism preventing killing of normal autologous cells may be provided by the 2B4 surface molecule, which, at early stages of NK cell differentiation, functions as an inhibitory rather than as an activating receptor.

Natural killer (NK) cells are capable of discriminating between normal cells and cells that have lost MHC class I expression as a result of tumor transformation or viral infection. This NK cell capability is the outcome of the concerted function of MHC class I-specific receptors that inhibit cytotoxicity upon recognition of their MHC ligands and of triggering receptors that induce NK cells to kill as a result of their interaction with non-MHC ligands.

In humans, the inhibitory receptors include the Ig-like killer inhibitory receptors (KIR) specific for HLA-A, -B, or -C, the CD94/NKG2A heterodimer, specific for HLA-E and the ILT2/LIR1, characterized by a broad specificity for different HLA class I molecules (1–3). The receptors responsible for NK cell activation in the process of target cell lysis have remained elusive until recently. They represent a heterogeneous molecular family composed of NKp30 (4), NKp44 (5, 6), and NKp46 (7, 8), all confined to NK cells, which have been termed collectively "natural cytotoxicity receptors" (NCR) (9, 10). Another triggering receptor, the NKG2D, is expressed by both NK cells and cytolytic T lymphocytes (11, 12). The 2B4 surface molecule, mostly functioning as an activating coreceptor, is expressed by NK cells and by a subset of cytolytic T lymphocytes (13, 14), whereas the NKp80, another recently identified coreceptor, is essentially confined to NK cells (15).

The surface expression of various inhibitory receptors during the NK cell development from CD34$^+$ cell precursors has recently been analyzed, but no such information is available for the various triggering receptors and coreceptors involved in natural cytotoxicity. Studies on the NK cell development have been based mostly on the analysis of progenitors derived from fetal liver, cord blood, thymus, or adult marrow. These progenitors, characterized by the CD34$^+$ Lin$^-$ surface phenotype, differentiated into mature NK cells when cultured under appropriate conditions, including the use of different cytokines and stromal cells (16–23). For example, in the presence of IL-2 as the only added cytokine, the development of precursors toward NK cells depended on their direct contact with stromal ligands (24). On the other hand, the requirement for stroma could be bypassed when NK cell progenitors were cultured in the presence of a mixture of cytokines including IL-15, IL-7, Flt3-L (FL), and stem cell factor (SCF) (16–23). In the present study we addressed the question of the sequential surface expression and function of the various triggering or inhibitory receptors during human NK cell differentiation from CD34$^+$ Lin$^-$ cell precursors.

Methods

Monoclonal Antibodies (mAbs). The following mAbs were used in this study: JT3A (IgG2a anti-CD3), c127 (IgG1 anti-CD16), KD1 (IgG2a anti-CD16), c218 (IgG1 anti-CD56), FS280 (IgG2a anti-CD56), A6–220 (IgM anti-CD56), BAB281 (IgG1 anti-NKp46), KL247 (IgM anti-NKp46), AZ20 (IgG1 anti-NKp30), F252 (IgM anti-NKp30), Z231 (IgG1 anti-NKp44), KS38 (IgM anti-NKp44), ON72 and BAT221 (IgG1 anti-NKG2D), MA152 (IgG1 anti-NKp80), MAR206 (IgG1 anti-CD2), XA185 (IgG1 anti-CD94), Z270 (IgG1 anti-NKG2A), EB6 (IgG1 anti-p58.1), GL183 (IgG1 anti-p58.2), PAX180 (IgG1 anti-p50.3), FS172 (IgG2a anti-p50.3), Z27 (IgG1 anti-p70), Q66 (IgM anti-p140), F278 (IgG1 anti-LIR1), PP35 (IgG1 anti-2B4), S39 (IgG2a anti-2B4), MA344 (IgM anti-2B4), MA127 (IgG1 anti-NTB-A), and ON56 (IgG2b anti-NTB-A) were produced in our laboratory. J4–57 (IgG1 anti-CD48) and RMO52 (IgG2a anti-CD14) mAbs were purchased from Coulter. PE-HPCA2 (anti-CD34) and TU145 (IgM anti-CD48) mAbs were purchased from Becton Dickinson.

Purification of Human Cord Blood CD34$^+$ Progenitors and Culture Conditions. Umbilical cord blood (UCB) samples from full-term newborns were collected at the Department of Gynaecology Istituto Giannina Gaslini (Genoa, Italy) upon informed consent of the mothers. UCB mononuclear cells were obtained by Ficoll–Hypaque density gradient centrifugation. CD34$^+$ cells were separated from mononuclear cells by using the MACS system (Miltenyi Biotec, Auburn, CA). Cells obtained in this manner were routinely ≥98% pure. CD34$^+$ Lin$^-$ cells were

Abbreviations: NK cells, natural killer cells; NCR, natural cytotoxicity receptors; UCB, umbilical cord blood; FL, fetal liver tyrosine kinases 3 (Flt3)-ligand; SCF, stem cell factor; KIR, killer Ig-like receptors; XLP, X-linked lymphoproliferative disease; SH2D1A, Src homology 2 (SH2) domain-containing protein 1A; SHP-1, SH2-containing phosphatase 1; E/T, effector-to-target cell ratio; PE, phycoerythrin.

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cultured in 24-well plates at a concentration of 1–3 × 10^4 in 1.5 ml of MyeloCult H5100 medium (StemCell Technologies, Vancouver) supplemented with 10% human AB serum (ICN), 5% FCS (Euroclone, Wetherby, U.K.), and the indicated cytokines. These progenitors were cultured in the presence of MS-5 (21), a murine stromal cell line that was grown to confluence in 24-well plates before use. Purified recombinant human IL-15, FL, SCF, and IL-7, purchased from PeproTech (London), were used at 20 ng/ml.

Phenotypic Analysis and Cytolytic Activity of NK Cells. At regular intervals during culture the phenotype of growing cells was analyzed by using a FACScan one- or two-color fluorescence cytofluorimetric analysis (7, 8). Purified $CD56^+$ NK progenitors were tested for cytolytic activity in a 4-h ^{51}Cr-release assay against human ($Fc\gamma R^+$ K562 cell line, EBV+ Raji Burkitt lymphoma, FO-1 melanoma), or murine ($Fc\gamma R^+$ P815 mastocytoma cell line) targets (4, 5). In other experiments immature NK cells were tested under the same conditions against purified immature myelomonocytic cells that differentiate *in vitro* from $CD34^+$ precursors together with NK cells. The concentrations of the various mAbs added were 0.5 μg/ml for redirected killing or 10 μg/ml for masking experiments. The effector-to-target cell (E/T) ratios are indicated in the figure legends.

Reverse Transcription–PCR Analysis. Total RNA was extracted from NK immature precursors or mature NK population by using RNAClean (TIB-MOLBIOL, Genoa, Italy), and oligo(dT)-primed cDNA was prepared by the standard technique. The SH2D1A cDNA (632 bp) was amplified with the following primers: 5′ CAG CGG CAT CTC CCT TG (SH2D1A-3 ORF frw) and 5′ TTT CAA AGC TCC TCA CTA TG (SH2D1A-4 ORF rev). Amplification was performed for 30 cycles: 30 s at 94°C, 30 s at 55°C, 30 s at 72°C. The set of primers SHP1-up (from nucleotide 155 to 173): 5′-TTC GGA TCC AGA ACT CAG G and SHP1-down (from nucleotide 736 to 755): 5′-TGC AAA CTC TCA AAC TCC TC were used to amplify the 601 bp of SHP-1 cDNA (30 cycles PCR: 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C). Both PCR reactions were performed with Taq-Gold (Perkin–Elmer/Applied Biosystems) after preactivation of 15 min at 95°C. The PCR products were resolved into a 0.8% agarose gel.

Results

Early Expression of Triggering NK Receptors by Immature NK Cells Differentiating from $CD34^+$ Lin^- Cell Precursors. Highly purified $CD34^+$ Lin^- cell populations isolated from UCB did not express NK cell markers, including CD56, NKp46, and NKp30 (Fig. 1*a*). These populations containing hemopoietic cell precursors were cultured with IL-15, IL-7, FL, and SCF in the presence of MS-5, a murine stromal cell line shown to support and to amplify the human NK cell differentiation from $CD34^+$ cell progenitors (21). Under these culture conditions $CD34^+$ Lin^- cells (10^4 cells per well) underwent proliferation and progressively lost the expression of CD34 surface antigen (not shown). After 20 days of culture ≈4–5 × 10^5 cells per well were recovered. A fraction of these cells were $CD56^+$ (Fig. 1*b*), whereas most of the remaining cells were differentiating toward the myelomonocytic cell lineage as indicated by the expression of CD33 and CD14 (not shown). The $CD56^+$ cell subset did not express other NK cell markers, including NKp46 (Fig. 1*b*), NKp30, NKG2D, CD16, CD94, NKp80 or KIR, T (CD3) and B cell (CD19) markers, and myeloid cell markers (CD14 or CD33) (not shown). Although, at this time, the surface density of CD56 molecules was low, a progressive increment of fluorescence intensity occurred at later stages of differentiation. Thus, after 30 days of culture, a fraction of cells were $CD56^{bright}$ (Fig. 1*b*). The acquisition of the $CD56^{bright}$ phenotype preceded the expression of

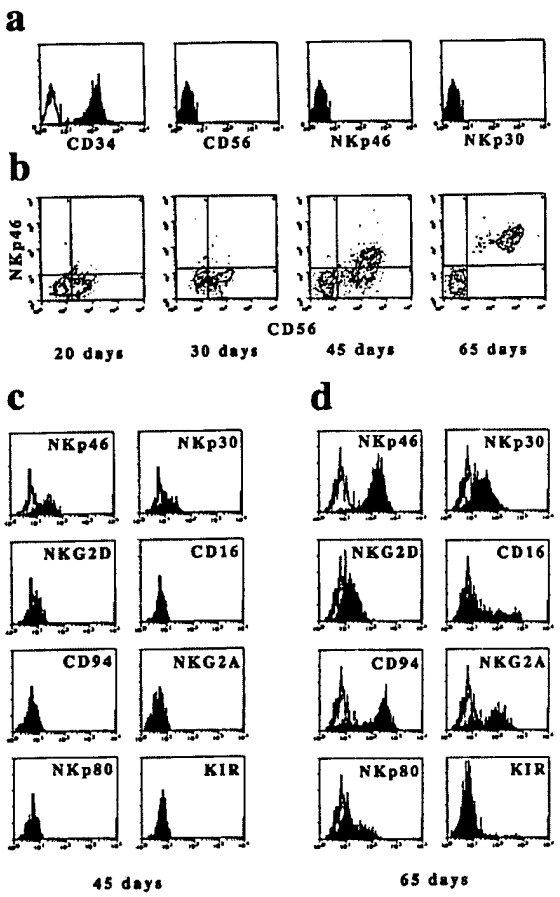

Fig. 1. Progressive acquisition of NK receptors by $CD34^+$ Lin^- cell precursors cultured in the presence of cytokines and stroma. (*a*) Freshly isolated $CD34^+$ Lin^- cord blood cells were stained with A6-220 (anti-CD56), KL247 (anti-NKp46), and F252 (anti-NKp30) mAb followed by phycoerythrin (PE)-conjugated isotype-specific goat anti-mouse second reagents and PE-HPCA2 (anti-CD34) mAb. White profiles represent cells stained with the second reagent alone. (*b*) $CD34^+$ Lin^- cord blood cells were cultured in the presence of IL-15, IL-7, FL, and SCF in direct contact with MS-5 cell line and analyzed by two-color immunofluorescence and fluorescence-activated cell sorter analysis for the expression of NKp46 in combination with CD56 molecules at the indicated time intervals. The contour plots were divided into quadrants representing unstained cells (*Lower Left*), cells with only red fluorescence (*Upper Left*), cells with red and green fluorescence (*Upper Right*), and cells with only green fluorescence (*Lower Right*). (*c*) Immature NK cells derived from $CD34^+$ Lin^- cord blood cells were cultured for 45 days. Gated $CD56^+$ cells were analyzed for the expression of the indicated NK cell markers. (*d*) Gated $CD56^+$ cells at day 65 were analyzed for the expression of the indicated NK cell markers. KIR were detected by the combined use of the following mAbs: GL183 (anti-p58.2/p50.2), EB6 (anti-p58.1/p50.1), Z27 (anti-p70), Q66 (anti-p140), and PAX180 (anti-p50.3). White profiles represent cells stained with the secondary reagent alone.

NKp46 (Fig. 1*b*) and NKp30 (not shown). Thus, at day 45 of culture, most $CD56^{bright}$ cells coexpressed these activating receptors (Fig. 1*c*). At this stage, both receptors were expressed at low surface densities, but their fluorescence intensities progressively increased with time. Indeed, cells with a NKp30/$NKp46^{bright}$ phenotype could be detected after 65 days of culture (Fig. 1 *b* and *d*). Remarkably, at day 45, CD94/NKG2A and other HLA class I-specific inhibitory receptors were still undetectable (Fig. 1*c*). Indeed, the expression of CD94/NKG2A occurred at later stages and >60% of cells expressed this inhibitory receptor at day 65, whereas KIR^+ cells represented a minor subset (2–8%) (Fig. 1*d*). At this stage a larger cell fraction expressed ILT2/LIR1 (15–25%) (not shown), CD16 (30–40%),

and NKp80 (30–40%) (Fig. 1*d*). The expression of NKG2D was slightly increased as compared with day 45. Similar results were obtained in seven independent experiments from different UCB.

Acquisition of Natural Cytotoxicity During NK Cell Development. Next, we assessed the cytolytic activity of NK cells undergoing differentiation in parallel with the expression of triggering receptors. In this context, previous data showed that mature NK cells express variable surface densities of NCR and that this expression correlated with the magnitude of the natural cytotoxicity (25). Thus, NCR^{bright} NK cells were characterized by high cytolytic activity, whereas the NCR^{dull} ones were poorly cytolytic against most NK-susceptible target cells (25). As illustrated above, the NK cell progenitors undergoing differentiation in culture displayed slowly progressing increases in the surface densities of NKp30 and NKp46. This observation allowed us to analyze the cytolytic activity of NK cells at discrete stages of maturation. Immature NK cells ($CD56^+$) were tested against the K562 target cell line ($Fc\gamma R^+$ $CD48^-$). K562 cells are characterized by a high susceptibility to lysis by mature NK cells, and they are also suitable for redirected killing analysis because of the surface expression of FcγR. As shown in Fig. 2*a*, NK cell progenitors acquired cytolytic activity in a stage-related fashion. Thus, after 30 days, cells characterized by the $CD56^+$, $NKp30^-$, $NKp46^-$, CD94/$NKG2A^-$ surface phenotype (see Fig. 1*b*) did not lyse K562 cells. According to their NCR^- phenotype, no cytotoxicity could be triggered in redirected killing in the presence of (IgG1) anti-NKp46 (Fig. 2*a*) or anti-NKp30 (not shown) mAbs. These data indicate that immature NK cells that do not express NCR are not cytolytic against classical NK susceptible target cells. At day 45 the surface expression of low amounts of NCR (NCR^{dull}) coincided with the appearance of low levels of cytotoxicity against K562 (Fig. 2*a*). At day 65, the higher surface density of NKp46 and NKp30 receptors coincided with a sharp increase in the magnitude of both spontaneous and anti-NCR mAb-induced cytolytic activity that reached levels comparable with mature NK cells (Fig. 2*a*).

To assess directly whether the cytolytic activity acquired by developing NK cells was indeed dependent on NCR expression and function, the cytolytic assay was also performed in the presence of anti-NCR mAbs of IgM isotype, to avoid cross-linking by the Fcγ receptors expressed on K562 target cells. The presence of these mAbs allows disruption of the interaction between the NCR and their ligands. Indeed, the combined masking of NKp46 and NKp30 by specific mAbs resulted in a sharp inhibition of lysis of K562 mediated by immature (day 45) NK cells (Fig. 2*b*). At this stage, the contribution of NKG2D to the lysis of K562 was negligible because of the low expression of this molecule on immature NK cells. In mature NK cell populations derived from peripheral blood (Fig. 2*c*) and in immature NK cells at day 65 (not shown) both NCR and NKG2D contributed to the NK-mediated recognition and lysis of K562 cells.

The cytolytic activity of immature NK cells was tested also against additional NK-susceptible target cells (e.g., the HLA class I-negative human melanoma FO-1) (4). Again, the cytotoxicity against these targets paralleled the expression of NKp30 and NKp46 and was inhibited by specific mAbs (not shown).

Altogether, these results indicate that the acquisition of cytolytic activity by developing NK cells parallels the surface expression of NKp30 and NKp46. Maximal cytotoxicity against suitable targets was detectable at late stages of maturation when NK cells had expressed both NCRs and NKG2D and their cytolytic activity was mediated by all these triggering receptors, as occurs in mature NK cells.

2B4 Displays Inhibitory Rather Than Activating Function in Immature NK Cells. The expression of triggering receptors (NCR) before the HLA-specific inhibitory receptors poses the question of why

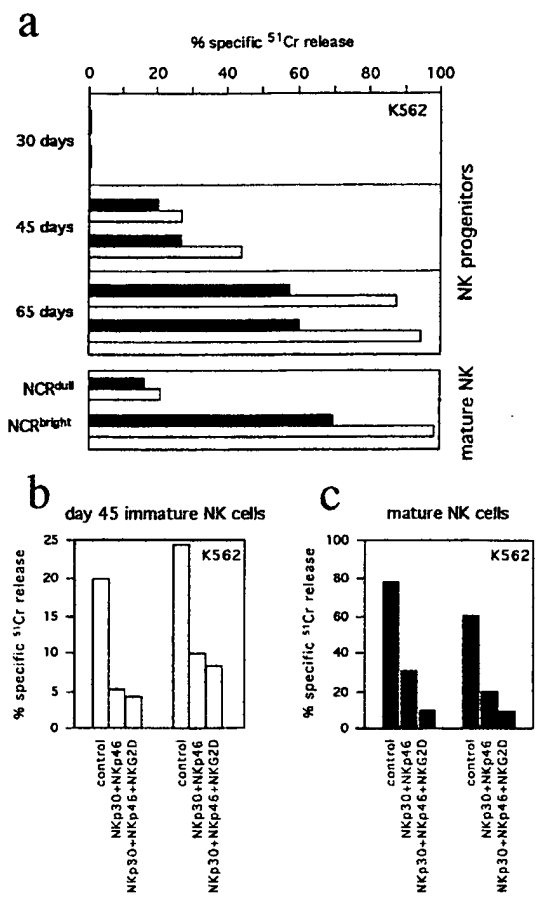

Fig. 2. Progressive acquisition of cytolytic activity by $CD34^+$ Lin^- cell precursors and involvement of NCR and NKG2D molecules. (*a*) Purified $CD56^+$ NK progenitors at various times were analyzed for cytolytic activity against the NK-susceptible $Fc\gamma R^+$ target cells K562 either in the absence (■) (spontaneous cytotoxicity) or in the presence of BAB281 (IgG1, anti-NKp46) (□) mAb (redirected killing). At each interval the cytolytic activities of two distinct immature NK populations (derived from different UCB) are shown. The cytolytic activity mediated by NK progenitors was compared with that of NCR^{dull} and NCR^{bright} mature IL-2-activated NK cell populations. The E/T ratios were 4:1 (NK progenitors and NCR^{dull} mature NK population) and 2:1 (NCR^{bright} mature NK population). (*b*) Two distinct purified $CD56^+$ immature NK cell populations at day 45 (□) were analyzed for cytolytic activity against the K562 target cell line either in the absence or in the presence of the indicated mAbs. To block the receptors, without inducing redirected killing effects, the mAbs used in these experiments were $F(ab')_2$ (anti-NKG2D) or IgM (anti-NKp30 and anti-NKp46). (*c*) For comparison the same mAb-mediated blocking experiments were performed with two different IL-2-activated mature NK populations (■). The E/T ratios were 4:1 for NK progenitors and 2:1 for IL-2-activated mature NK populations.

immature NK cells do not kill normal surrounding cells. This could be explained by the presence of a still undefined self-reactive inhibitory receptor. Another distinguished possibility would be the involvement of surface receptors such as 2B4 mediating signals of opposite sign depending on their association with different intracytoplasmic polypeptides (27). This possibility was taken into consideration because, in preliminary experiments, the mAb-mediated engagement of 2B4 in a redirected killing assay against P815 ($Fc\gamma R^+$) murine target cells resulted in inhibition of cytolysis mediated by immature NK cells. Notably, in mature NK cells, human 2B4 is involved in triggering the NK-mediated natural cytotoxicity (13, 14) and this effect depends on its association with a small cytoplasmic molecule termed Src homology 2 (SH2) domain-containing protein [SH2D1A; or signaling lymphocyte activation molecule

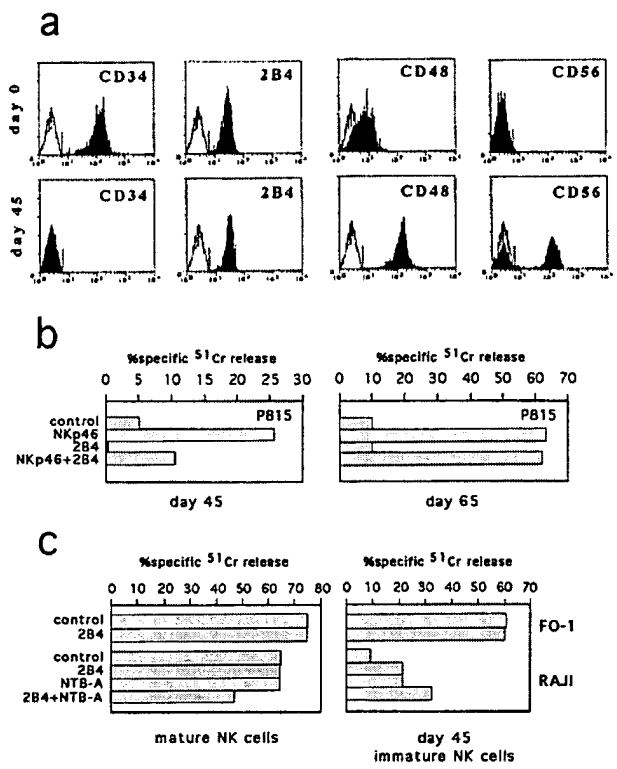

Fig. 3. Expression and function of 2B4 molecule during NK development. (*a*) $CD34^+$ Lin^- cord blood cells freshly isolated or cultured for 45 days in the presence of both cytokines and stroma were stained with S39 (anti-2B4), TU145 (anti-CD48), or FS280 (anti-CD56) mAbs followed by PE-conjugated isotype-specific goat anti-mouse second reagents or with PE-HPCA2 (anti-CD34) mAb. White profiles represent cells stained with the secondary reagent alone. (*b*) Purified $CD56^+$ immature NK cells cultured for 45 days (*Left*) or 65 days (*Right*) were assessed for cytolytic activity in a redirected killing assay against the $Fc\gamma R^+$ P815 target cell line either in the absence or in the presence of the indicated (IgG1) mAbs. The E/T ratio was 4:1. (*c*) Mature NK cells (*Left*) or purified NK progenitors cultured for 45 days (*Right*) were assessed for cytotoxicity against $CD48^-$ (FO-1, melanoma) or $CD48^+$ (Raji, Burkitt lymphoma) target cells in the presence or in the absence of mAb specific for the indicated molecules. In these experiments of receptor blocking, the mAb used was $F(ab')_2$ or of IgM isotype. The E/T ratios were 4:1 for mature NK cells and 15:1 for NK cell precursors.

(SLAM)-associated protein (SAP); refs. 26 and 27]. This molecule apparently competes with SHP phosphatases for binding to the cytoplasmic tail of 2B4 (26). Remarkably, in patients with X-linked lymphoproliferative disease (XLP), lacking SH2D1A molecules because of critical mutations in the SH2D1A-encoding gene, the cross-linking of 2B4 leads to inhibition rather than activation of cytotoxicity (27).

To evaluate the possibility that 2B4 may play a role during the NK cell maturation, we analyzed its expression and function at different stages of development. Cytofluorimetric analysis revealed that 2B4 was surface expressed already in freshly isolated $CD34^+$ cell progenitors (Fig. 3*a*), and its expression remained unchanged during the whole NK cell differentiation process. The 2B4 ligand, represented by CD48 (28), was expressed at low density in fresh $CD34^+$ cells but, upon culture, was up-regulated in all differentiating cells, including both immature NK cells and bystander myelomonocytic cell precursors (Fig. 3*a*). Immature NK cell populations cultured for 45 days were assessed for cytolytic activity in a redirected killing assay against P815 target cells that are relatively resistant to spontaneous NK-mediated killing (Fig. 3*b*) (10). Although mAb-mediated cross-linking of NKp46 induced triggering of cytolytic activity, cross-linking of 2B4 failed to induce cytotoxicity but rather exerted an inhibitory effect. This inhibitory effect could be demonstrated better in experiments in which mAb-mediated cross-linking of NKp46 and 2B4 was induced simultaneously. These data indicate that, in immature NK cells (similar to NK cells from XLP patients), the engagement of 2B4 can lead to down-regulation of the NCR-mediated triggering. This was further confirmed in experiments in which immature NK cells were analyzed for cytotoxicity against $CD48^-$ (FO-1) or $CD48^+$ (EBV^+ Raji Burkitt lymphoma) target cells (Fig. 3*c*). Different from $CD48^-$ target cells, $CD48^+$ cells were resistant to lysis by immature NK cells whereas they were highly susceptible to lysis by mature NK cells (Fig. 3*c*). mAb-mediated blocking of the 2B4/CD48 interactions could restore, at least in part, the cytolytic activity of immature NK cells against $CD48^+$ targets (Fig. 3*c*). This effect could be further incremented by the simultaneous mAb-mediated blocking of NTB-A, a surface molecule that has recently been shown to bind SH2D1A in normal NK cells (29). Because even under these conditions the reconstitution of cytotoxicity mediated by immature NK cells was not complete, it is possible to speculate that additional receptors delivering inhibitory signals may be implied in the recognition of ligands expressed by Raji cells. These receptors might even be represented by still-undefined members of the SH2D1A-binding family.

The inhibitory effect mediated by 2B4 at day 45 was not detected further at day 65 of culture (Fig. 3*b*), but, as expected, in mature NK cells the mAb-mediated cross-linking of 2B4 resulted in triggering of the cytolytic activity (not shown). A similar 2B4-mediated phenomenon in NK cells from XLP patients reflected the lack of SH2D1A molecules (27). To verify whether immature NK cells also were characterized by a defect of SH2D1A, cells cultured for 45 days (surface phenotype: $NKp46^+$, $NKp30^+$, $NKG2A^-$, $LIR1^-$, KIR^-) were analyzed for the expression of SH2D1A transcripts. Reverse transcription–PCR analysis failed to reveal SH2D1A transcript in these immature NK cells, whereas SHP-1 transcript was detected in all samples analyzed (Fig. 4*a*). Although not shown, expression of SH2D1A could be detected at later stages of NK differentiation (i.e., day 65).

Inhibitory 2B4 Molecules Allow Immature NK Cells to Spare Bystander Cell Precursors. In an attempt to verify whether the inhibitory function of 2B4 could play any physiologic role in self-tolerance during NK cell maturation, we analyzed the cytolytic activity mediated by NK precursors at day 45 against autologous purified immature myelomonocytic cells. As mentioned above, these cells differentiate together with NK cell precursors in cultures derived from $CD34^+$ Lin^- cells and are characterized by high surface expression of CD48, i.e., the 2B4-specific ligand (see Fig. 3*a*). As shown in Fig. 4*b*, immature NK cells (day 45), although lacking HLA class I-specific inhibitory receptors, were poorly cytolytic against purified (autologous) myelomonocytic cells. Their cytolytic activity could be significantly rescued (>50%) by mAb-mediated blocking of 2B4. Controls included polyclonal NK cell populations derived from an XLP patient or from a normal individual. Also, in the case of the XLP patient, the NK-mediated lysis of myelomonocytic precursors was significantly increased on mAb-mediated blocking of 2B4 molecules (Fig. 4*b*). In contrast normal (allogeneic) mature NK cells were highly cytolytic, and lysis was not modified under the same experimental conditions. Although not shown, the NK-mediated lysis of myelomonocytic precursors was NCR dependent as indicated by mAb-mediated blocking experiments. These data suggest that immature NK cells may spare surrounding precursors cells by a mechanism involving 2B4 as in XLP patients. However, immature NK cells displayed some degree of cytolytic activity against myelomonocytic precursors even in the absence of mAb-mediated blocking of 2B4 (Fig. 4*b*). This observation suggests

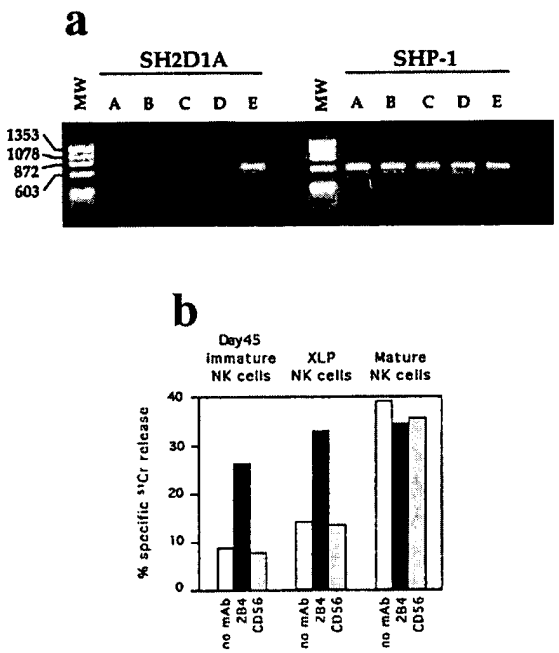

Fig. 4. Lack of SH2D1A transcripts in immature NK cells and the role of 2B4 in self-tolerance toward bystander cell precursors. (*a*) Reverse transcription–PCR analysis of SH2D1A (*Left*) and SHP-1 (*Right*) transcripts was performed on total RNA isolated from four representative immature (day 45) NK cell populations (lanes A–D) or from a mature NK cell population (lane E). Lengths (in base pairs) of the principal bands of *Hae*III-digested phage ϕX174 DNA are indicated on the left. (*b*) Purified NK cell precursors (day 45) were assessed for cytotoxicity against autologous myelomonocytic precursors that, under the above-described culture conditions, differentiate together with NK cells. The cytolytic effect was analyzed either in the absence or in the presence of the indicated mAb of IgM isotype. Controls include mature polyclonal NK populations from XLP patient A (27, 29) or from a normal donor. The E/T ratio was 12:1 for immature NK cells, 5:1 for XLP-NK cells, and 3:1 for normal NK cells.

that immature NK cells are not completely turned off by the inhibitory signals generated by 2B4, as also demonstrated by the ability of NK cells to undergo proliferation and maturation in a microenvironment containing $CD48^+$ cells. It is conceivable that the incomplete down-regulation of NK cell function may allow the delivery of signals needed for NK precursors to progress toward a mature phenotype. These data provide an interesting explanation of how the early expression of 2B4, accompanied by a late expression of SH2D1A, may lead to the negative control of natural cytotoxicity during NK cell development.

Discussion

Taken together, our data provide additional insight into the process of human NK cell maturation. A remarkable finding was the early expression of triggering receptors mediating natural cytotoxicity. Because this event preceded the expression of HLA class I-specific inhibitory receptors, it posed a serious problem about to how to avoid the NK cell-mediated attack to surrounding cells at the site of NK cell maturation. Importantly, we show that the 2B4 receptor, expressed early during NK cell maturation, may provide this necessary fail-safe device.

Human $CD34^+$ cell progenitors, cultured *in vitro* in the presence of suitable cytokines, differentiated into NK cells, most of which expressed the CD94/NKG2A inhibitory receptor (19, 30, 31). Almost no KIR expression could be detected in these cultures, unless suitable stromal cells providing undefined signals were used to support the NK cell differentiation (22, 23). Similar results were obtained in mice, in which the expression of Ly49 inhibitory receptors required both stroma and cytokines (32, 33). Thus, according to these reports, the NK cell self-tolerance at a relatively early stage of human or murine NK cell maturation would be ensured by the expression of CD94/NKG2A that precedes that of human KIR or murine Ly49. Our present study, allowing a better dissection of the early events occurring during the human NK cell differentiation, indicated that the current models of self-tolerance during NK cell development should be reconsidered. Although the education processes that finally give rise to a (self-tolerant) NK cell repertoire are still poorly defined, they may require signals provided by both triggering and inhibitory receptors that are sequentially expressed at the cell surface. Of the different models that have been proposed to explain the integration between triggering and inhibitory receptors during the NK cell development (reviewed in ref. 34), one is compatible with our present data. This model that is based, at least in part, on recent studies in mice, suggests that the first receptors to be expressed during NK cell maturation are (still undefined) triggering receptors. Their engagement with self-ligands, however, would not result in induction of cytotoxicity but would be limited to the delivery of signals responsible for the subsequent expression of MHC-specific inhibitory receptors (CD94/NKG2A and, subsequently, KIR or Ly49). In line with this model, we show that NKp30 and NKp46, two major triggering receptors involved in natural cytotoxicity in humans, are expressed before the CD94/NKG2A inhibitory receptor. However, immature $NKp30^+$ $NKp46^+$ $CD94/NKG2A^-$ NK cells displayed cytolytic activity in response to stimuli acting on triggering receptors. Thus, because NKp30 and NKp46 receptors were functional, one may ask how, in such immature (but cytolytic) NK cells, they can deliver signals resulting in cell differentiation without affecting self-tolerance. A possible explanation may be provided by studies on MHC class I knockout mice and on human HLA class I-deficient individuals (35–38). In these cases, the engagement (and the function) of MHC-specific inhibitory receptors (consistently present in NK cells from MHC-deficient subjects) is impaired because of the low levels of expression of MHC ligands. The resulting NK cells, however, do not kill autologous cells, thus implying the existence of a still-undefined fail-safe mechanism. Two models have been proposed to explain the self-tolerance of NK cells derived from class I-deficient subjects. According to the first model, lack of autoreactivity may be consequent to the down-regulation of stimulatory receptors involved in NK cell triggering. The second model proposes a role for a still-undefined inhibitory receptor for non-MHC ligands. Recent studies from our laboratory support the second hypothesis because both the expression and the function of the major triggering NK receptors (NKp30, NKp46, and NKG2D) was apparently normal in HLA class I-deficient individuals (TAP2 $-/-$) (39). A similar scenario could be envisaged for developing NK cells analyzed in the present study. Indeed, these cells, although expressing triggering receptors, still lack MHC class I-specific inhibitory receptors. Thus, the inhibitory receptor or receptors capable of extinguishing their cytolytic activity should be acquired very early during NK cell differentiation and its expression should be independent on signaling by stimulatory receptors. We provide evidence suggesting that 2B4 represents a suitable candidate to fulfill this role, rendering self-tolerant potentially autoreactive immature NK cells. Our finding that, in immature NK cells, 2B4 displays inhibitory function allowed understanding of how it is possible to block potentially harmful cells. The actual key is represented by the late expression of the SH2D1A molecule. Thus, in immature NK cells, 2B4 would replace class I-specific inhibitory receptors until the stage at which they are surface expressed. The expression of 2B4 precedes that of all of the presently known triggering NK receptors. Thus, 2B4 is expressed on $CD34^+$ precursors, and its expression is maintained through the whole differentiation process toward mature NK cells. Because it is expressed in early hematopoietic precursors, it is possible that

2B4 may play a more general regulatory role in the process of hematopoiesis, possibly by blocking the induction of unwanted function(s) during the early steps of cell differentiation.

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Enhancement of Natural Killer Activity by an Antibody to CD44[1]

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ABSTRACT. In this study, we examined the in vitro effect of an anti-CD44 mAb, S5, on NK function using canine PBMC as effectors. S5 enhanced NK activity in a dose-dependent and rapid fashion as did IM7, another anti-CD44 mAb that recognizes a common epitope(s) on CD44. Other anti-CD44 mAb (Hermes-1 and S3) that recognize epitopes distinct from S5 and IM7 had a variable effect on NK activity. The activation of increased killing by S5 only occurred when NK-sensitive targets were used, suggesting that lymphokine-activated killer cells were not being induced. Antibody-dependent cell cytotoxicity was not the mechanism involved in the augmentation of NK activity, nor was it Fc receptor-dependent, inasmuch as S5 $F(ab')_2$ was able to increase NK activity. F(ab') fragments of S5 were equivalent to intact S5 in their ability to stimulate NK activity, demonstrating that cross-linking of CD44 was not a necessary component of stimulation, and that nonspecific agglutination of target and effector cells was not occurring via the two F(ab) arms. The enhancement of NK function was monocyte-independent and mediated by radioresistant cells, indicating that the antibody enhanced NK cells directly. This finding would suggest that CD44 can direct a transmembrane signal for NK cell activation. *Journal of Immunology,* 1993, 150: 812.

A canine model was previously developed to examine the mechanism(s) of marrow graft rejection. Inasmuch as radioresistant host cells with a morphology of LGL[3] and NK-like activity may be involved in rejection of dog leukocyte-nonidentical grafts (1), efforts were directed at generating a mAb against these host cells (2). This mAb, designated S5, when given i.v. at 0.2 mg/kg/day for 7 days before TBI and marrow transplantation abrogated this rejection (2). By FACS analysis, S5 binds most canine PBMC (>90%) and cross-reacts weakly with human PBMC. As demonstrated by sequential immunoprecipitation and tryptic digestion, S5 recognizes CD44 (3).

CD44 is a ubiquitous glycoprotein molecule found in different organs and tissues and exists in different isoforms with diversified molecular weight (4) and numerous functional roles (5). It is found in different subpopulations of hematopoietic cells (4) and has been previously described as Pgp-1 (6, 7), In(Lu) related p80 (8), Hermes (9), ECMR-III (10), and Hutch 1 (11). Antibodies against CD44 have been shown to increase human peripheral T cell activation induced through CD2 or CD3 TCR pathways (12, 13), block T cell proliferation stimulated by anti-CD3 mAb (14), block lymphohematopoiesis in long term bone marrow culture (15), trigger the release of human monocyte TNF (16) and IL-1 (12, 16), block lymphoid cell adhesion (17, 18), and augment the cytolytic activity of CTL (19).

Until now, the effect of mAb recognizing CD44 on NK function has not been reported. NK cells are defined as nonadherent, nonphagocytic $CD3^-$ LGL that are capable of killing target cells without prior priming (20–24). Unlike cytotoxic T cells, this killing is also observed in target cells with no or very low expression of MHC class I (25). NK activity is known to be increased by IL-2 (26, 27), IFN

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[3] Abbreviations used in this paper: LGL, large granular lymphocytes; ADCC, antibody dependent cell cytotoxicity; TBI, total body irradiation; CTAC, canine thyroid adenocarcinoma cell.

(28, 29), NK stimulatory factor (30), TNF (31), mAb to porcine NK and murine NK cells (32, 33), and chemical agents (34, 35). In our present study, we found that S5 increases NK activity in vitro in canine PBMC in a dose-dependent and rapid fashion.

Materials and Methods

Effector cell population

Healthy beagles were used as donors of blood for PBMC. PBMC were prepared by density gradient centrifugation on Ficoll-Hypaque (density, 1.074) and washed three times with Waymouth medium (Irvine Scientific, Santa Ana, CA) + 1% penicillin streptomycin + 2% nonessential amino acids (Gibco, Grand Island, NY) and finally resuspended in RPMI (Irvine Scientific) + 1% nonessential amino acids + 1% L-glutamine (Gibco) + 1% sodium pyruvate (Gibco) + 10% FCS (Hyclone, Logan, UT) that had been heat-inactivated at 56°C for 30 min. PBMC were then used as effectors.

mAb

The murine anti-CD44 mAb, S5 (subclass IgG1), was previously generated against canine cells (2). S3 (murine IgG2b), another anti-CD44 mAb, was also generated against canine cells (2) (our unpublished observations). The other anti-CD44 mAb IM7 (rat IgG2b) and Hermes-1 (rat IgG2a) were generated against mouse and human cells, respectively, and have been described previously (18, 36). The antibody Thy-1 (F-3-20-7, murine IgG1), previously generated against canine T lymphocytes from lymph node, recognizes canine Thy-1 Ag (37) and was used as a positive isotype control antibody. The negative control mAb, 31A (murine IgG1), does not cross-react with canine cells.

Cell lines

The cell lines used were CTAC (38), K562 (a human erythroleukemia cell line) (39), CT45S (a canine lymphoblastic cell line) (38), and Daudi (a human Burkitt lymphoma cell line) (29).

In vitro irradiation

PBMC were exposed to irradiation for different periods of time with a ^{137}Cs source that emits radiation at a rate of 813 rad/min. The cells were subsequently used for the NK assays.

NK assay

CTAC (40, 41) was used to quantitate enhancement of NK activity in dogs. One-tenth ml of target cells (CTAC) were incubated with 0.1 ml (about 300 μCi) of $Na^{51}CrO_4$ (New England Nuclear, Boston, MA) at 37°C for 60 min, washed once, and incubated with RPMI + 10% FBS + 1% nonessential amino acids + 1% penicillin-streptomycin + 2% L-glutamine + 1% sodium pyruvate, at 4°C for 30 min and washed twice. Target cells were adjusted to 5×10^4 cells/ml and added to 96 U-bottomed wells (Costar) at 0.1 ml/well. The effector cells were preincubated with various mAb and added to the wells at an E:T ratio of 60:1 in triplicate wells. These were incubated at 37°C for the required time (15 to 18 h) and centrifuged at $100 \times g$ for 2 min. One-tenth ml of the supernatant was then harvested using a multipipettor (Titertek), and the radioactivity was determined using a gamma scintillation counter (Packard). Percent specific lysis was determined using the formula:

$$\% \text{ Specific lysis} = \frac{\text{Experimental cpm} - \text{spontaneous release cpm}}{\text{Maximum release cpm} - \text{spontaneous release cpm}}$$

Maximum release was determined using 100 μl of 1.0% Nonidet P-40 to lyse the labeled targets.

ADCC assay

Target cells were incubated with respective mAb after being radiolabeled (as described above) and washed three times with medium, resuspended at 10^5 cells/ml, and added to the wells at 0.1 ml containing 0.1 ml of the effector cells. The supernatant was harvested as above.

Preparation of $F(ab')_2$

Inasmuch as S5 is a mouse IgG1 mAb, we used preactivated papain (Sigma) rather than pepsin to prepare $F(ab')_2$ (42). Briefly, the antibody was dialyzed in 0.1 M acetate/0.003 M EDTA, pH 5.5, buffer overnight. Papain was activated by incubation with 0.05 M free-base cysteine (Sigma) for 30 min at 37°C and passed over a PD-10 column (Pharmacia no. G-25) that had been equilibrated with sodium acetate/EDTA buffer to remove excess cysteine. The amount of papain in each eluate fraction was calculated as $OD_{280}/2.5$ to give mg preactivated papain/ml. The papain was then added to the dialyzed antibody at a ratio of 1:20 (wt:wt) and incubated for about 6 h at 37°C. The reaction was stopped by adding iodoacetamide to a final concentration of 0.03 M. The digestion product was dialyzed in PBS and passed over a protein A column to remove any undigested antibody and Fc fragments. It was then analyzed by SDS-PAGE with Coomassie blue stain and gel-filtration HPLC.

Preparation of F(ab') of S5

Papain was dissolved in digestion buffer (PBS containing 0.02 M EDTA and 0.02 M cysteine) at 0.1 mg/ml papain. Papain (100 μl) was then added to 100 μl of 2 mg/ml S5 to

give an enzyme:antibody ratio of 1:20. This was incubated at 37°C for 10 h. The reaction was stopped by adding 20 µl of 0.3 M iodoacetamide. The crude preparation was dialyzed against PBS at 4°C overnight, passed through protein A column, and redialyzed against PBS. The purity of the F(ab') preparation was assessed using SDS-PAGE and gel filtration-HPLC, and was found to be free of undigested antibody and other byproducts.

Flow cytometry

Cells (1×10^6) were incubated with 10 µg/ml of various mAb for 30 min at 4°C, washed three times with Hanks' balanced buffered saline with 2% FCS and 5 mM sodium azide, and incubated with 50 µl of 1:100 FITC-conjugated goat anti-mouse antibody (Biochemica, Indianapolis, IN) for another 30 min at 4°C. Cells were then washed three times, resuspended in 0.3 ml of paraformaldehyde, and analyzed on an Epics flow cytometer. Controls consisted of cells treated with FITC-conjugated goat anti-mouse antibody alone and irrelevant control mAb, followed by secondary mAb.

Monocyte depletion

Cells (2×10^8) were incubated in a 14-cm-diameter plastic tissue culture dish for 1 h at 37°C and transferred to a second Petri dish for an additional hour to remove adherent cells that were not depleted in the first incubation. The nonadherent cells were then loaded onto a 10-cm nylon wool column that had been preincubated with supplemented Waymouth and incubated at 37°C for 1 h. Nonadherent cells were then eluted using 20 ml media at a rate of 60 drops/min. The cells in the collected eluant were washed twice and used in the NK assay, FACS analysis, and cytospin preparation.

Morphology

The morphology of canine PBMC obtained from Ficoll-Hypaque gradient and that from monocyte-depleted populations were further examined using cytospin preparations. Cells (2×10^5) were spun at 700 rpm for 7 min and stained with Wright-Giemsa or nonspecific esterase to differentiate monocytes.

Results

Characterization of NK cell activation

S5 was observed to augment NK activity in a dose-dependent fashion in canine PBMC (Fig. 1) using an E:T ratio of 60:1. The same results were seen at both higher and lower E:T ratios. To achieve a good response, we subsequently used 10 µg/ml of S5 and other mAb for effector cell incubation. Figure 2 compares the different anti-CD44 mAb with respect to their ability to increase NK activity.

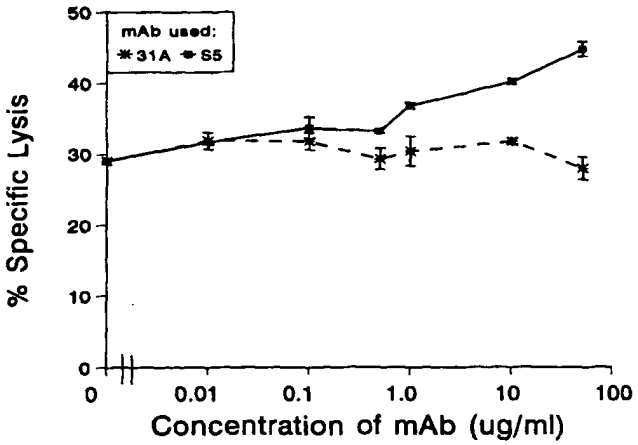

FIGURE 1. Dependence of NK activation on antibody concentration. The quantitation of NK activity (as measured by percent specific lysis) relative to concentration of mAb is compared for PBMC (0.01 to 50 µg/ml). The enhancement of NK activity by S5 was dose dependent. The E:T ratio used in this and the subsequent experiments was 60:1 unless otherwise stated. This and the subsequent data are presented as mean ± SE of triplicate wells in the assay and confirmed for reproducibility.

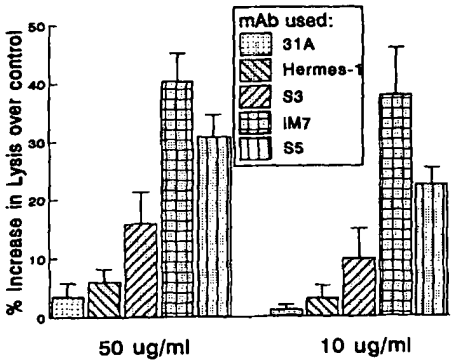

FIGURE 2. Comparison of NK activation using different anti-CD44 mAb. Different anti-CD44 mAb (Hermes-1, S3, IM7, and S5) were studied with respect to NK activation of PBMC. The results were expressed as percent increase of specific lysis over untreated control and are the average of nine representative experiments. S5 and IM7 were able to enhance NK activity at 50 µg/ml and 10 µg/ml, whereas S3 increased NK activity only variably at 50 µg/ml.

Only S5 and IM7 were able to consistently enhance NK activity in PBMC. Hermes 1, another anti-CD44 mAb tested, did not have any effect, even when saturating concentrations of up to 100 µg/ml were used (data not shown). mAb S3, on the other hand, had a mild effect of increasing NK activity at higher doses (50 µg/ml). Unlike S5 and IM7, this enhancement was not consistently seen in all experiments. The mAb 31A was used as a negative control antibody. The activation of NK activity by S5 could be discerned even at a lower E:T ratio of 15:1, although the extent was reduced (data not shown). For the sake of simplicity, all of our results are presented at E:T ratio 60:1

(unless otherwise stated). We also performed the paired *t* test using 95% confidence on our accumulated data for both S5 ($n = 15$) and IM7 ($n = 9$) using an E:T ratio of 60:1 and a mAb concentration of 10 μg/ml, and found that the difference between NK function of untreated PBMC and S5 or IM7-treated PBMC was significant ($p < 0.0001$).

Kinetics of increased NK activity

The activation of NK activity, using a suboptimal concentration of S5 (1 μg/ml), reached the peak in just 1 h of preincubation (Fig. 3*a*). The increase in NK activity could be detected as early as the second hour of the NK assay (Fig. 3*b*), indicating a rapid enhancement effect. Target killing in the control experiments was not observed until the fourth hour. Employing different preincubation temperatures (4°, 20°, and 37°C), we were still able to demonstrate S5 enhancement of NK activity (Fig. 3*c*).

Specificity of enhancement

The enhancement effect could only be induced when NK-sensitive targets, but not NK-resistant targets, were used (Table I). S5 enhancement of NK activity in dogs could only be discerned using CTAC cells but not other cell lines (CT45S, Daudi, K562).

ADCC experiment

There was no enhancement in target cell killing mediated by ADCC (Fig. 4). In this assay, CTAC cells were first preincubated with the antibodies before exposure to the effector cells. ADCC measures the extent of effector cells killing target cells via antibody bridging either in conventional (direct) ADCC or reverse ADCC (where mAb binds to the effector and the Fc portion binds to the target).

$F(ab')_2$ experiment

S5 $F(ab')_2$ was generated using preactivated papain and analyzed and purified using SDS-PAGE and HPLC, and was found to be free of undigested antibody and breakdown products. Compared to the intact antibody, there was no decrease in the stimulation of NK activity with the $F(ab')_2$ fragments (Fig. 5). In fact, the extent of enhancement was comparable to that of the intact antibody. The Fc portion of S5 was, therefore, not required for the enhancement effect.

F(ab') experiment

S5 F(ab') fragments were generated using papain as described in *Materials and Methods*. The enhancement with F(ab') fragments was comparable to the intact antibody (Fig. 6), which provides evidence that cross-linking of CD44 was not a necessary component of this activation.

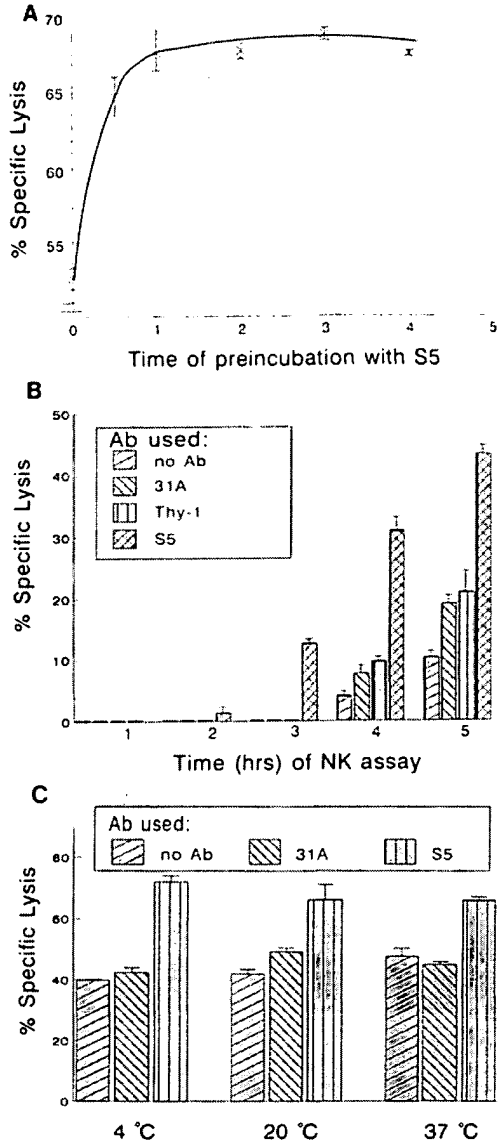

FIGURE 3. Characteristics of canine NK activation by S5. *a*, Using a suboptimal dose of S5 (1 μg/ml), effectors were preincubated with S5 for an increasing period of time (0 to 4 h). It took only 1 h of preincubation for the rate of killing to reach peak activity. *b*, NK activity was monitored for the first 5 h of NK assay. The kinetics of enhancement was rapid. At 2 h, the S5-treated cells manifested NK killing in contrast to controls that had no killing. *c*, The effectors were preincubated with mAb at different temperatures (4°C, 25°C, and 37°C) and their NK activity determined.

More importantly, these data rule out direct antibody-mediated binding of targets and effectors via the two F(ab') arms.

In vitro radiation of effectors

In the first experiment (Fig. 7*a*), PBMC were first incubated with mAb for 1 h, irradiated with 0 to 80 Gy, and used to set up a NK assay immediately. S5 enhancement of

Table I
Result of using different cell lines [a] with effector cells

Canine Effector Treatment	% Specific Lysis of Cell Lines:			
	CTAC	CT45S	K562	Daudi
No mAb	64.3 ± 0.8	4.1 ± 1.4	11.1 ± 1.0	1.3 ± 0.4
31A	65.5 ± 3.9	4.5 ± 0.9	11.3 ± 0.9	0.2 ± 0.8
Thy-1	67.9 ± 4.1	2.3 ± 0.9	9.7 ± 1.3	1.2 ± 0.3
S5	81.1 ± 3.6	1.8 ± 0.5	11.9 ± 1.4	3.8 ± 0.3

[a] CTAC is an NK-sensitive target, whereas CT45S, K562, and Daudi are NK-resistant targets and do not demonstrate the enhancement effect by S5. The E:T ratio used was 60:1. The incubation time for all the assays was 15 h.

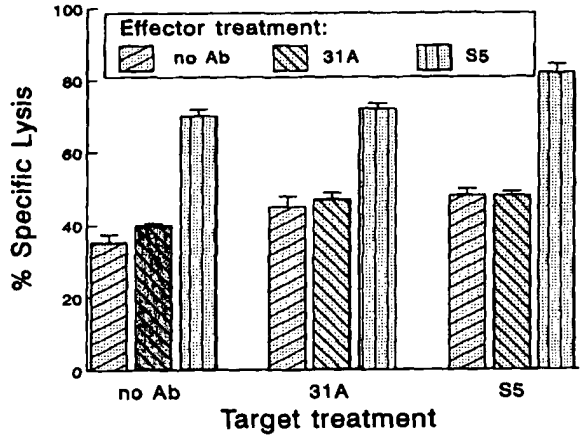

FIGURE 4. Study of role of ADCC in NK activation. CTAC cells were preincubated with 31A and S5 and exposed to effector cells that were also treated with the mAb. Only S5-treated effectors were able to induce an enhancement in NK activity in the S5-treated target. If ADCC was involved, an increase in activity of the control and 31A-treated effectors using S5-treated target would have been seen.

NK activity was still seen, although the extent of enhancement decreased with increases in radiation dose. In the second experiment (Fig. 7*b*), PBMC were first irradiated with 0 to 80 Gy, incubated with mAb for 1 h, and used in an NK assay immediately. In PBMC that were irradiated up to 80 Gy, cells were still present that could respond to S5 activation of NK activity. In the third experiment (Fig. 7*c*), PBMC were first irradiated, rested for 24 h to enrich for radioresistant cells, incubated with mAb for 1 h, and used in the NK assay. PBMC were enriched for NK cells as indicated by higher NK activity in the controls that received 9.2 Gy as compared to no irradiation (0 Gy). Again, the S5-treated cells were found to have enhanced NK activity relative to control, even after 9.2-Gy irradiation and rest for 24 h.

Monocyte depletion

Plastic adherence and nylon wool separation methods were used to deplete monocytes, and the nonadherent cells were found to contain less than 1% monocytes. Monocyte-depleted effector cells did not abrogate S5 enhancement of NK activity (Fig. 8). Subpopulations of monocyte-depleted PBMC were determined by FACS analysis (90° light scatter vs forward light scatter) and morphology of cytospin preparation using Wright-Giemsa and nonspecific esterase stain, inasmuch as mAb specific for canine NK cells are presently only being developed. The monocyte subpopulation (as determined by either method) was consistently found to comprise less than 1% of cells, whereas cells of LGL morphology made up 25 to 30%.

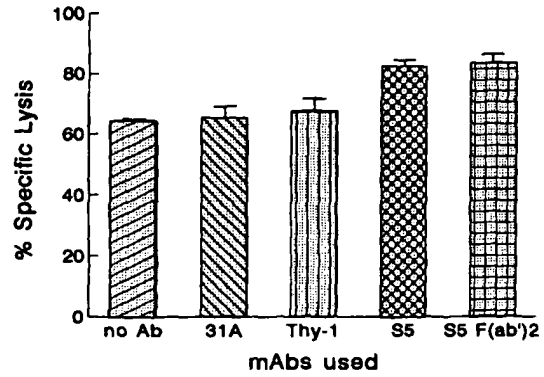

FIGURE 5. Effect of $F(ab')_2$ fragment of S5 on NK function. $F(ab')_2$ fragments of S5 were used in the NK assay. The concentration of mAb used was 10 μg/ml. An increase in NK activity, comparable to that of the undigested mAb, was documented for the $F(ab')_2$ fragment of S5. Thy-1 is a second isotype control mAb.

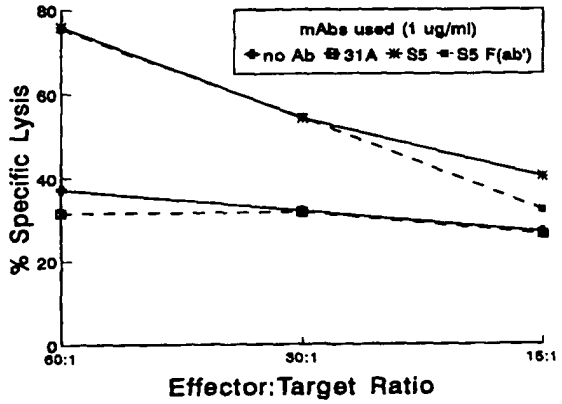

FIGURE 6. Effect of F(ab') fragment of S5 on NK function. F(ab') fragments of S5 were still able to augment NK activity of PBMC at a concentration of 1 μg/ml and E:T ratio of 60:1 to 15:1.

Discussion

In this paper, we report that certain anti-CD44 mAb enhanced NK activity in vitro in canine PBMC. Because the assays were performed in such a short period of time (<24 h), the cytolytic activity can be attributed to NK cells and not cytotoxic T cells that require about 4 to 7 days of priming. The activation of NK activity was consistently seen with two CD44 mAb, S5 and IM7, that recognize common epitope(s) on the CD44 molecule. It was previously demonstrated in blocking experiments that only S5

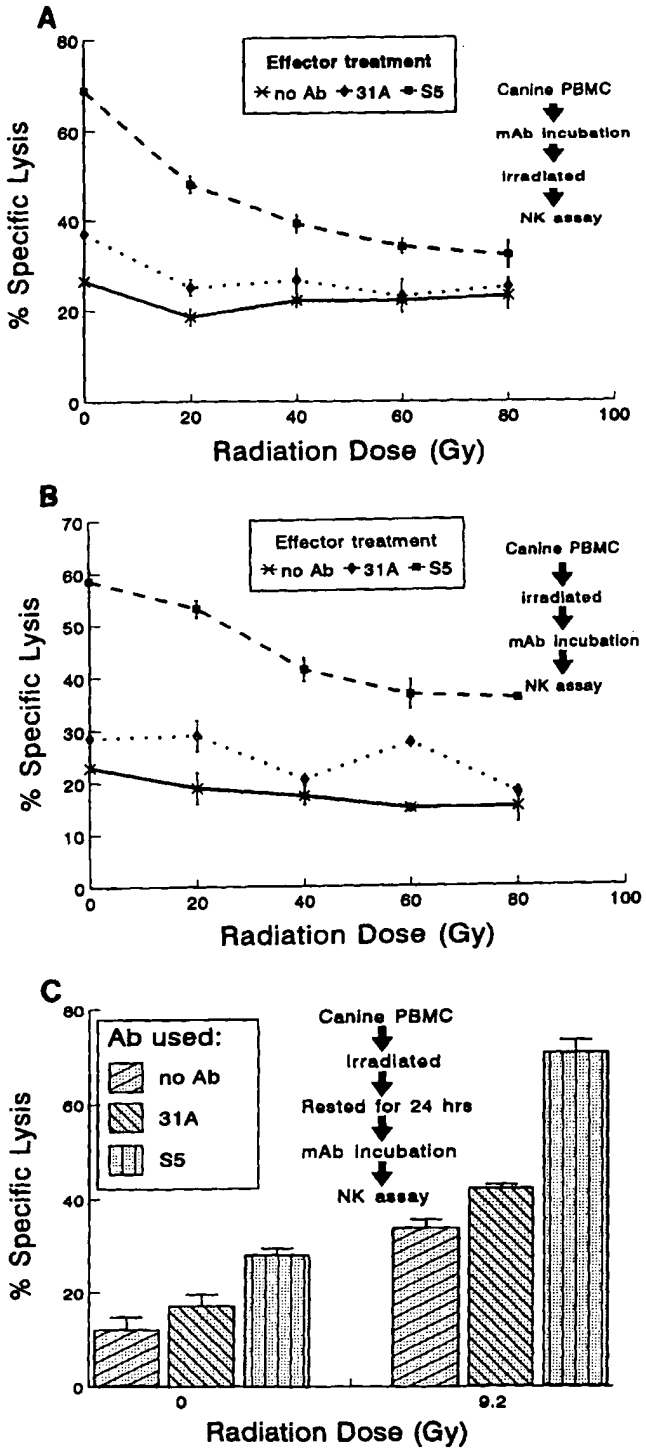

FIGURE 7. Study of the effect of irradiation on NK activation by S5. *a*, Effector cells were incubated with mAb for 1 h, irradiated from 20 to 80 Gy, and used in NK assays immediately. S5 was able to increase NK activity despite irradiation of effectors. *b*, Effector cells were irradiated, preincubated with mAb for 1 h, and used to set up NK assay immediately. The enhancement effect by S5 was not completely abrogated. *c*, Effectors were irradiated at 9.2 Gy and allowed to rest for 24 h. These were then preincubated with mAb for 1 h and used in NK assay. Effectors became enriched with radioresistant cells that were still able to respond to S5.

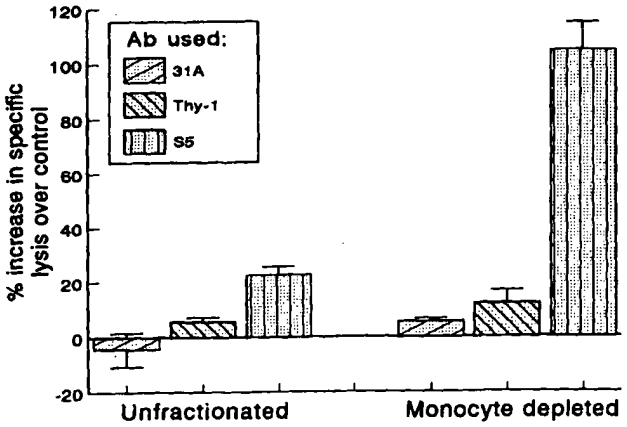

FIGURE 8. Monocyte depletion experiment. For comparison, these data were presented as percent increase over the control (no mAb) for the same group (unfractionated vs monocyte depleted). The monocyte-depleted sample that became enriched in LGL was very responsive to S5, showing a 100% increase in specific lysis over the control.

and IM7 blocked each others' binding to the CD44 molecule, whereas S3 (our unpublished observations) and Hermes-1 (3) did not. This would suggest that the binding of a specific epitope(s) on the CD44 molecule is sufficient to trigger a signal(s) necessary for NK cell activation. Whether CD44 is a physiologic molecule implicated in activation of NK cells, or whether it is a bystander that can turn on the normal signal transduction for NK cell activation, is unknown.

The increase in NK activity was very rapid and required only 30 min of preincubation with S5. These experiments would indicate that S5 is a potent stimulator of NK activity. The extent of stimulation of NK activity varied with different dogs (from 22.6 to 99.7% increase over control). This rapid killing could be the consequence of an increase in the number of activated NK cells or the result of an increase in the frequency of killing by activated NK cells (29). There was a general trend of dogs with lower NK activity responding to S5 better than those with higher NK activity, as indicated by a higher percentage increase in specific lysis (data not shown). The temperature of the mAb-effector cell incubation phase did not seem to affect the ability of S5 to increase NK activity, showing that Ag modulation was unlikely the predominant mechanism responsible for the phenomenon. Even in assays using serum-free medium (data not shown), S5 was still able to bring about similar effects, indicating that the S5-induced increase of NK activity was independent of extrinsic factors (such as cytokines present in the serum).

This enhancement effect was specific to CTAC, which is an NK-susceptible target cell line. When NK-resistant cells and K562, the NK target cell for humans but not for dogs, were used, this effect was not seen, even if the E:T ratio was increased from 60:1 to 120:1. This specificity

points to the fact that certain molecule(s) need to be present on the NK-sensitive target cell (43, 44), which may be a species-specific phenomenon. The ligand on K562, which is a human cell line, may not be recognizable by canine PBMC. Using human PBMC with K562 targets, S5 was able to induce NK activation, but this observation was variable between different individuals (data not shown). This may reflect the lower avidity of S5 to its ligand on human cells or a differential sensitivity of the target (K562) to the activated effector cell. Enhancement was restricted to K562, which excluded the involvement of lymphokines such as IL-2 in the activation, inasmuch as S5 treatment did not induce killing of Daudi cells (data not shown).

Because the target cells express CD44, it became necessary for us to investigate whether ADCC was the mechanism involved in the S5 enhancement effect (20, 21). Neither direct nor reverse ADCC (19) was present in our experiments, inasmuch as target cell incubation with S5 did not lead to an increase in cell lysis in the presence of untreated and 31A-treated effectors.

To show that increased NK activity was not dependent on the Fc portion of the antibody molecules as shown with other antibodies (32), we prepared $F(ab')_2$ fragments of S5. This was best achieved by using preactivated papain, which did not require as harsh a condition (pH 5.5) and produced less breakdown products (our unpublished observations) compared to using pepsin (42). When we used S5 $F(ab')_2$ in our assay, the enhancement effect was not eliminated. This provided additional evidence that the Ag binding site of the anti-CD44 mAb was responsible for NK cell activation, and that ADCC was not the mechanism.

To rule out whether nonspecific agglutination of effector cells to target cells was occurring, we carried out the experiments using F(ab') fragments of S5. Unlike intact mAb and $F(ab')_2$ fragments that have two binding sites, F(ab') fragments have only one Ag binding site and therefore cannot agglutinate an effector to a target. Our results in Figure 6 show that incubating canine PBMC with F(ab') fragments of S5 still produces the same effect, indicating that the effect is not the result of a nonspecific approximation of the target to effector cell. In addition, these data suggest that cross-linking of CD44 is not a necessary component of NK activation.

Inasmuch as it was well established that NK cells are radioresistant (23, 40), we expected NK cells to remain functional despite irradiation. The fact that activation of NK cells by S5 still occurred after exposure to high irradiation doses implied that S5-enhanced NK activity was independent of radiosensitive cells (such as T lymphocytes) and provided evidence that NK cells are directly involved. To help us to understand the original observation that S5 increases marrow engraftment, we incubated PBMC with S5, rested them for 24 h, and irradiated them before setting up a NK assay. We found that the S5-treated PBMC were more radiosensitive compared to the untreated PBMC control in some cases, whereas in other cases there were no observable differences. Because this was not a consistent finding, we examined NK function in a dog that was infused with S5 from day −7 through −2 before TBI in the same experimental model in which the initial observation of graft enhancement was made. In the PBMC that were obtained immediately after TBI, there was a significant decrease in NK function as compared to the PBMC that were obtained before TBI. In contrast, this decline of NK function was not seen after TBI in an untreated control animal. This finding is consistent with our observation that S5-treated PBMC were more radiosensitive (as indicated by a steeper slope on Fig. 7*a*) at lower radiation doses. If persistent host NK function is responsible for graft rejection as previously postulated (1), then pretreatment with S5 may abrogate graft rejection by activating NK cells in vivo and secondarily making them more susceptible to radiation kill.

Monocyte depletion of PBMC did not interfere with S5-induced enhancement of NK activity. It was necessary for us to rule out a possible involvement of monocytes, inasmuch as they had been previously shown to serve as accessory cells to NK activity by elaborating external stimuli (34). In fact, the extent of S5 increased in NK activity of the monocyte-depleted sample was greater than that of the unfractionated sample compared to their respective control, possibly implying additional mechanism(s) in the enhancement of canine NK activity. Other adherent cells (such as B and dendritic cells) would also have been removed by this treatment (45–47), which may have a negative regulatory control of activation. We have also consistently observed that the adherent cell fraction failed to respond to S5 (data not shown). Based on these data, we can exclude any significant involvement of B cells and monocytes in the observed phenomena. The only NK-like cell lines available are those with a LGL phenotype, but because they are maintained in culture with IL-2, they are more a model for lymphokine-activated killer cells. Because S5 does not induce lymphokine-activated killer function, we presently are designing strategies to obtain a highly purified NK cell preparation in sufficient numbers to confirm our findings.

Other reports have demonstrated that antibodies to CD44 can induce homotypic cell aggregation (48), induce hyaluronic acid binding to some $CD44^+$ cells lines (49), and stimulate the release of macrophage CSF (50), IL-1 (12, 16), and TNF (16) from monocytes. Our current finding that CD44 enhances NK activity further supports the multiple roles of CD44 in both cellular adhesion and activation.

Preliminary data are consistent with S5-facilitated secretion of a potent soluble cytotoxic factor as well as an

increase in conjugate formation between effector and target cells. These observations of an enhancement in conjugate formation between effectors and targets could be attributed to a conformational change of the CD44 molecule on the effector cell after mAb incubation, which enables more effective binding of CD44 to its ligand on target cells. Reported ligands for CD44 include hyaluronic acid (51), extracellular matrix proteins (collagen types I and VI, and fibronectin) (10), and mucosa addressing (52). Alternatively, mAb binding to CD44 potentially could affect other adhesion molecules such as the β_2 integrins (CD11a-c/CD18), which are known to be involved in binding of NK cells to target cells (53, 54). Interestingly, mAb to CD44 are known to trigger LFA-1 (CD11a/CD18)-mediated adhesion (55) and therefore may be involved in the enhancement of NK activity by S5. These mechanisms are currently being investigated in our laboratory. We have cloned canine CD44 (our unpublished observations) and presently are mapping the epitope that S5 recognizes to elucidate further the mechanism of graft enhancement at a molecular level.

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NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics

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Natural killer (NK) cells have not previously been precisely identified or characterized in cattle or any other ruminant species. We have generated a monoclonal antibody against bovine NKp46, which is expressed exclusively by NK cells in man. NKp46$^+$ cells comprised 1–10% of blood mononuclear cells in cattle, and did not stain with antibodies against CD3, CD4, TCR1, B cell or granulocyte markers. The majority of the NKp46$^+$ cells expressed CD2, and a variable fraction also expressed CD8. The tissue distribution of NKp46$^+$ cells in cattle was compatible with the tissue distribution of NK cells in other species. Bovine NKp46$^+$ cells had typical, large granular lymphocyte morphology, and proliferated vigorously in response to bovine IL-2 for a limited number of cell divisions. IL-2-activated NKp46$^+$ cells killed the bovine kidney cell line MDBK. This cytotoxicity was inhibited by preincubation with antibody against NKp46. In a redirected lysis assay, IL-2-activated NKp46$^+$ cells killed the FcγR$^+$ target cell line P815 after preincubation with antibody against NKp46. Together, these data indicate that bovine NKp46 is an activating receptor and demonstrate the existence of a subset of leukocytes in cattle that, in terms of surface markers, morphology and function, represent NK cells.

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1 Introduction

Natural killer (NK) cells are large granular lymphocytes with the ability to spontaneously lyse certain tumor cells, virally infected cells or normal, MHC disparate cells [1–3]. NK cells express both activating and inhibitory receptors. Many of these receptors recognize MHC class I or class I-like ligands, and the balance between activating and inhibitory receptors determines the final outcome of the interaction of NK cells with possible target cells [4]. NK cells also provide a link between innate and acquired immunity through production of cytokines and interaction with antigen-presenting cells [1, 5–7]. Although bovine NK-like cells responding to mycobacteria-infected dendritic cells have been described [8], and cells with natural cytotoxicity have been found in cattle and sheep [9–11], the lack of mAb specifically recognizing NK cells has hampered the characterization of these cells in ruminants. Recently, genes encoding several bovine NK receptors have been identified [12, 13]. NKp46, previously characterized in primates and rodents, is a type I transmembrane glycoprotein with two extracellular C2-type Ig-like domains [14–17]. In the human, NKp46 is an activating receptor expressed exclusively by NK cells [18], and is involved in NK cell-mediated lysis of several targets including antigen-presenting cells [7]. The transmembrane region contains an arginine residue thought to interact with the FcRγ chain [14, 18], which contains immunoreceptor tyrosine-based activation motifs (ITAM). This region is conserved between the bovine, rodent and primate NKp46.

We here report the generation of an mAb against bovine NKp46. This mAb is used to characterize a subset of bovine leukocytes that, in terms of surface markers, morphology and functional characteristics, represent NK cells. We describe a method for isolation and culture of bovine NK cells, and demonstrate that bovine NKp46 activates cytotoxicity.

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2 Results

2.1 Generation of an mAb against bovine NKp46

A soluble fusion protein consisting of the extracellular region of NKp46 and the hinge and Fc regions of mouse IgG2b was generated and used to immunize mice. Hybridomas were screened for reactivity against 293T cells transfected with an NKp46-FLAG construct. One clone was isolated and termed AKS1. This clone produced an mAb of the IgG1 isotype that specifically recognized bovine NKp46 (Fig. 1A). In Western blot analysis of 293T cells transfected with NKp46-FLAG, AKS1 weakly stained a band of ~37 kDa and also a faint band of ~47 kDa. These bands were also stained with an anti-FLAG antibody (Fig. 1B). Immunoprecipitation with AKS1 of surface-biotinylated IL-2-activated NKp46$^+$ cells (see below) revealed a band of ~47 kDa both under reducing and non-reducing conditions (Fig. 1C), suggesting that bovine NKp46 is expressed as a monomer. The observed molecular mass is comparable with human NKp46. The theoretical mass of the unglycosylated NKp46 polypeptide is 32.2 kDa [13]. The difference is likely due to glycosylation.

2.2 NKp46 is expressed on a cell population with an NK cell phenotype

Two-color flow cytometric analysis was used to investigate surface expression of NKp46 on leukocytes in blood and selected tissues from 6–24-month-old animals. NKp46 was expressed on 1–10% of blood mononuclear cells. The NKp46$^+$ population was CD4$^-$, TCR1$^-$, and negative for the γδ T cell marker WC1, suggesting they were not a subset of T cells (Fig. 2A). The NKp46$^+$ population was further negative with mAb against B cells and granulocytes, respectively. Although a small fraction of the NKp46$^+$ cells expressed MHC class II, this population was CD14$^-$, suggesting that it did not represent monocytes. The NKp46$^+$ population was largely CD2$^+$, and between 4% and 15% of the NKp46$^+$ cells expressed CD8. NKp46$^+$ bovine leukocytes thus share key surface marker characteristics with NK cells in other species [19, 20]. NKp46$^+$ cells were found in liver, lung and spleen. Significant numbers of NKp46$^+$ cells were not present in the thymus or retropharyngeal lymph nodes, but between 1.3% and 3.9% of mesenterial lymph node cells expressed NKp46. While the majority of NK cells in liver, lung and blood expressed CD2, the majority of NKp46$^+$ cells in mesenterial lymph nodes and to a lesser degree in spleen were CD2$^-$ (Fig. 2B). NKp46$^+$ cells from all organs tested were negative for CD4, TCR1, WC1 and MHC class II, and did not stain with mAb against B cells or granulocytes (data not shown).

2.3 Isolation and culture of NKp46$^+$ cells

NKp46$^+$ cells were purified from PBMC by incubation with AKS1 mAb and positive selection with immunomagnetic beads. Culture in the presence of recombinant bovine IL-2 led to the up-regulation of CD25 expression within 1–3 days, and to cell proliferation. Cultures could typically be maintained between 7–14 days, yielding 10- to 20-fold increases in cell numbers. Human recombinant IL-2 did not induce up-regulation of CD25 or proliferation. Proliferating NKp46$^+$ cells grew as nonadherent cells with prominent lamellipodia (Fig. 3A). However, shortly after isolation, cells from some animals were adherent for several hours. In Giemsa-stained cytospin preparations, IL-2-activated NKp46$^+$ cells as well as

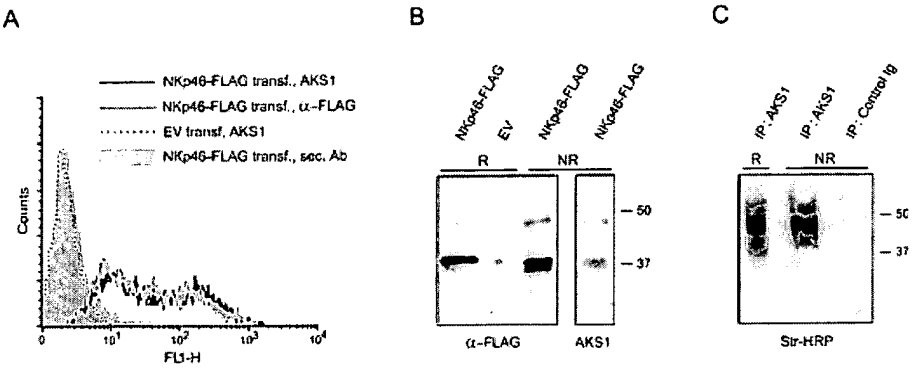

Fig. 1. (A) Flow cytometric analysis of 293T cells transfected with an NKp46-FLAG construct or empty vector (EV), and stained with supernatant from the AKS1 hybridoma or an anti-FLAG mAb. (B) Western blot of 293T cells transfected with an NKp46-FLAG construct or empty vector (EV) incubated with a rabbit anti-FLAG polyclonal Ab or the AKS1 mAb. (C) Immunoprecipitation of surface-biotinylated IL-2-activated NKp46$^+$ cells with AKS1 or an irrelevant isotype-matched control antibody. R: reducing conditions, NR: non-reducing conditions. Relative molecular masses in kDa are indicated.

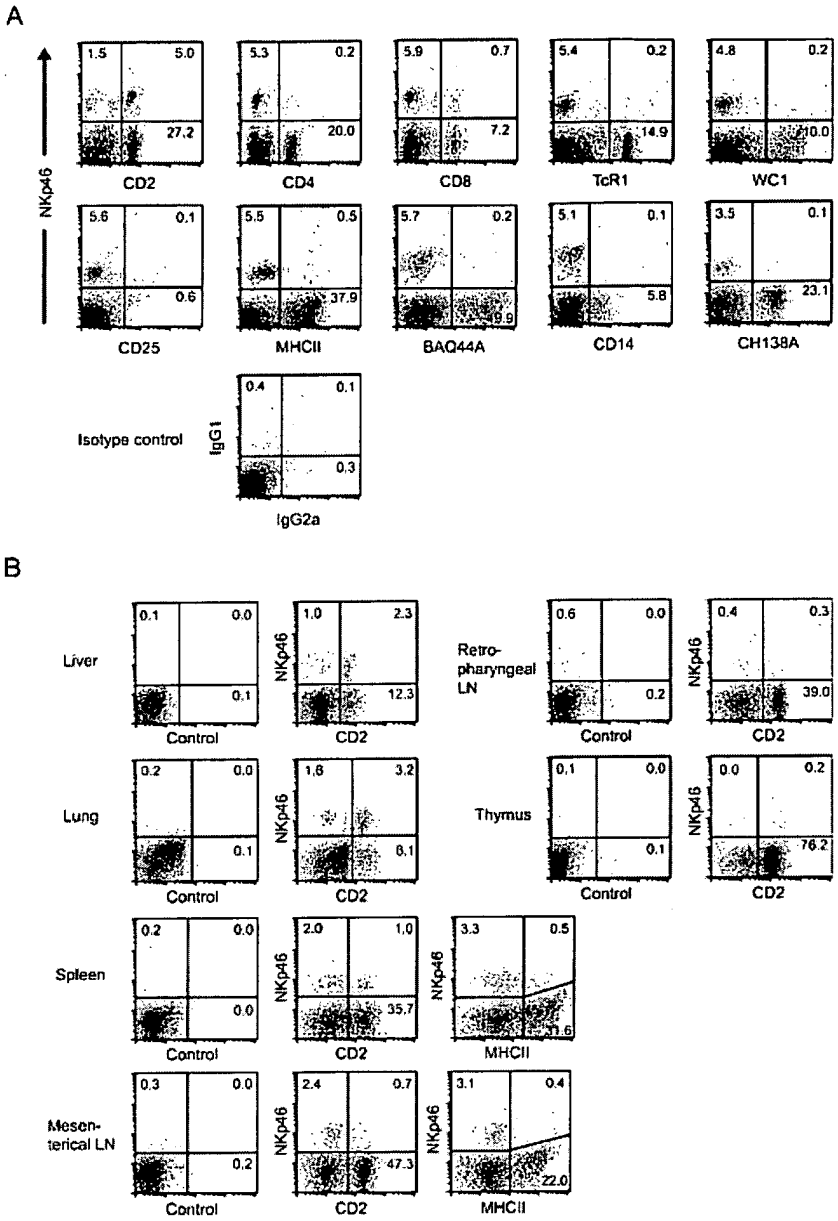

Fig. 2. Two-color flow cytometric analysis of NKp46 expression. (A) Blood cells stained with the AKS1 mAb and mAb against selected leukocyte markers. WC1 is a marker for a subset of γδ T cells; BAQ44A stains a major subset of B cells (B2); CH138A stains granulocytes. A forward/side scatter gate was set to include viable mononuclear cells, except for the granulocyte marker staining, where a larger gate also included viable granulocytes. (B) Cells from the indicated tissues stained with AKS1 and mAb against CD2 or MHC class II. The results shown are representative for experiments with eight (A) and five (B) different animals, respectively. LN: Lymph node.

freshly isolated NKp46$^+$ cells from blood were large granular lymphocytes, containing acidophilic granules polarized to one side of the cell (Fig. 3B). From all donors, 99.5% of IL-2-activated NK cells displayed a uniform NKp46^{bright}, CD3$^-$ phenotype (Fig. 4A, B). While the majority of NK cells in blood expressed CD2, the proportion was lower following IL-2 culture. In contrast, the level of CD8 expression was increased, and showed a continuous expression level from negative to brightly positive. Double labeling with CD8α and CD8β was not feasible due to steric interference between the two mAb. However, CD8α was more frequently expressed than CD8β, suggesting that some cells expressed a CD8α/α homodimer, while the majority expressed theCD8α/β heterodimer. CD8 was expressed on both CD2$^+$ and CD2$^-$ cells (Fig. 4B).

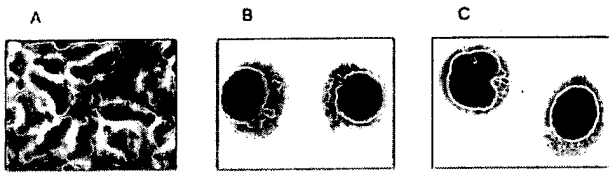

Fig. 3. (A) Phase-contrast microscopy image of NKp46$^+$ cells cultured in IL-2 for 6 days. (B) Giemsa-stained cytospin preparation of NKp46$^+$ cells cultured in IL-2 for 8 days and (C) Giemsa-stained cytospin preparation of NKp46$^+$ cells positively selected from blood.

2.4 Bovine NKp46$^+$cells spontaneously lyse tumor cells

In 4-h cytotoxicity assays, IL-2-activated NKp46$^+$ cells from one donor efficiently lysed the murine tumor targets YAC-1 and P815, as well as the human leukemic cell line K562 (Fig. 5A). A second donor showed lower levels of cytotoxicity, whereas six other animals did not show significant cytotoxicity against these cell lines. However, after preincubation with mAb against NKp46, effector cells from all eight donors induced efficient lysis of the FcγR-expressing cell line P815 even at low effector to target ratios. Preincubation with mAb against CD2 or CD8 did not induce lysis (Fig. 5B). Lysis of FcγR$^-$ target cells (YAC-1) was not induced by antibody against NKp46 (data not shown). Moreover, NKp46$^+$ cells from all animals tested killed the bovine kidney cell line MDBK

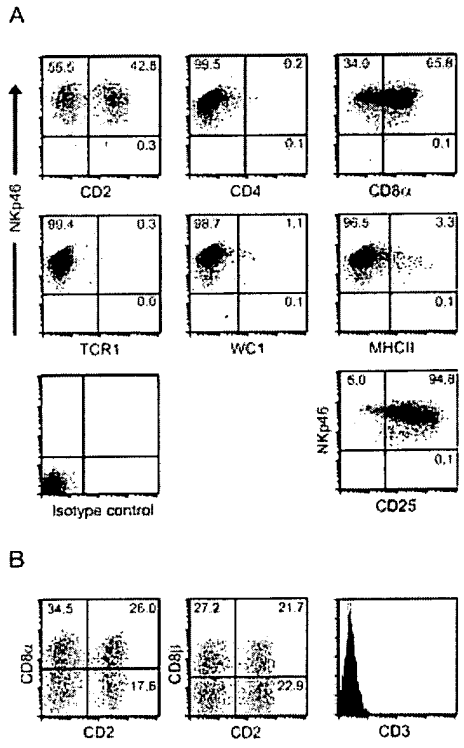

Fig. 4. Flow cytometric analysis of bovine IL-2-activated NKp46$^+$ cells. (A) Two-color staining with the AKS1 mAb and mAb against selected leukocyte markers. WC1 is a marker for a subset of γδ T cells. (B) Double staining with anti-CD2 mAb and mAb against CD8α or CD8β; and (far right) histogram displaying surface expression of CD3. The results shown are representative for experiments with five different animals.

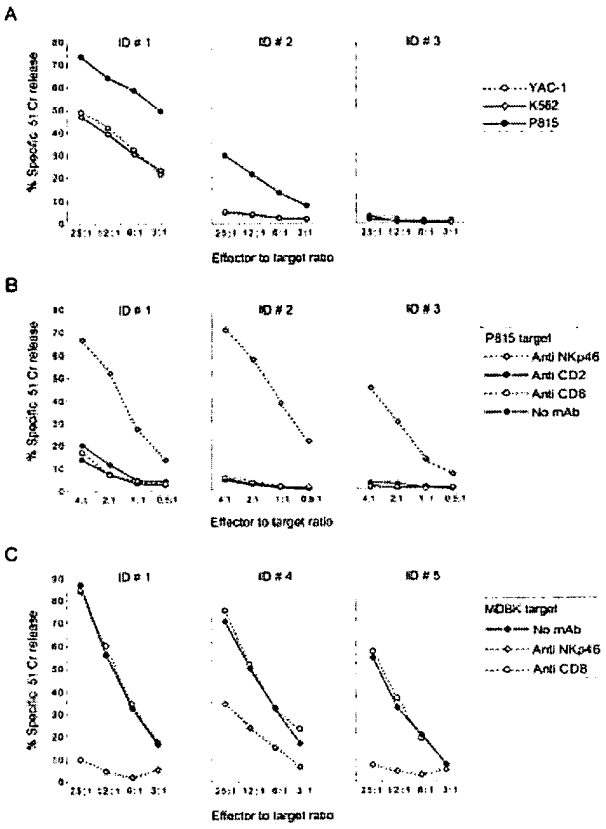

Fig. 5. (A) IL-2-activated NKp46$^+$ cells from eight different animals were tested for spontaneous cytotoxic activity against murine and human tumor cell lines in a 4-h ^{51}Cr-release assay. Effector cells from one of the animals (ID #1) showed cytotoxic activity against all three cell lines tested, while cells from a second animal (ID #2) only lysed P815 targets. Six other animals tested did not show spontaneous cytotoxic activity as exemplified by ID #3. Results shown are representative for at least two independent IL-2 cultures from each animal. (B) Redirected lysis assay. IL-2-activated NKp46$^+$ cells from the indicated animals were preincubated with the anti-NKp46 mAb AKS1 or mAb against CD2 or CD8 (all of IgG_1 isotype) and used as effector cells against the FcγR$^+$ tumor target P815 in a 4-h ^{51}Cr-release assay. The results shown are representative for all eight animals tested. (C) IL-2-activated NKp46$^+$ cells from all donors tested efficiently lysed the bovine kidney cell line MDBK, and preincubation with mAb AKS1 reduced this activity. Results shown are representative for at least two analyses from seven individual donors.

(Fig. 5C). Thus, NKp46$^+$ cells from all donors had the capacity for natural cytotoxicity. Together with the morphology and flow cytometry data, these functional data indicate that bovine NKp46$^+$ leukocytes represent NK cells.

Lysis of MDBK cells was reduced following preincubation of effector cells with mAb against NKp46, although to varying degrees in cultures from different animals (Fig. 5C), suggesting that MDBK cells express a ligand for NKp46. Together with the redirected lysis data, this also indicates that the NKp46 receptor activates cytotoxicity in bovine NK cells.

3 Discussion

In this study, we have generated an mAb against bovine NKp46 and used this mAb to define a subset of leukocytes in blood and other organs that functionally as well as phenotypically represent NK cells. This definition relies on several observations: (a) the majority of the NKp46-positive cells expressed CD2, and a variable fraction also expressed CD8, corresponding to the characteristics of NK cells in other species [19, 20]. Moreover, B cells, T cells, monocytes and granulocytes did not express NKp46. In concurrence with this, NKp46 has been shown to be specifically expressed by NK cells in the human [18]. (b) The tissue distribution of NKp46$^+$ cells in cattle is compatible with the tissue distribution of NK cells in mouse, rat and man [21, 22]. (c) Bovine NKp46$^+$ cells proliferate vigorously in response to IL-2 for a limited number of cell divisions, as is the case for NK cells in other species [19, 22]. (d) NKp46$^+$ cells had a typical NK cell morphology in culture as well as on Giemsa-stained cytospins. (e) IL-2-activated NKp46$^+$ cells had the capacity for spontaneous killing of bovine, murine and human target cell lines. We thus conclude that most or all bovine NK cells express NKp46. We have not detected NKp46 expression on other cell types than NK cells in cattle. Previously, cell surface markers such as CD16, CD56, CD94 and NKR-P1 have been used to define NK cells in man, mouse and rat. Antibodies against bovine orthologues of these markers are presently not available, and NK cells specifically express none of these markers. Based on our results, NKp46 should be regarded as a valid marker for bovine NK cells.

While there were almost no NKp46$^+$ cells present in the retropharyngeal lymph nodes, a small population of NKp46$^+$ cells could be detected in the mediastinal lymph nodes. Because the majority of these cells were CD2$^-$, they most likely did not represent contamination from blood NK cells present in the prepared tissue. Although significant numbers of NK cells are not believed to reside in lymph nodes under normal conditions, they have been reported to accumulate at these sites after regional challenges [23]. This may be an enduring state in the intestine of healthy farmed cattle, as used in this study. Subpopulations of human NK cells have been described that express CCR7 and have the ability to home into secondary lymphoid organs through high endothelial venules [24].

Considerable variation was observed between donors with regard to the level of spontaneous cytotoxicity towards the murine and human target tumor cell lines tested. The level of NK cytotoxicity against these targets for each donor remained unchanged between independent IL-2 cultures. Moreover, the cytotoxic potential was intact and similar between NK cells from all donors, as demonstrated by preincubating with anti-NKp46 mAb in a redirected lysis assay. This suggests that the difference in cytotoxicity is not due to individual differences in cytotoxic potential, but rather to different receptor repertoires between different donors. IL-2-activated cultures from all bovine donors expressed NKp46 at a similar level, in contrast to observations with human NK cells [25]. Thus, the difference in cytotoxicity was not due to different levels of NKp46 expression.

In contrast to the murine and human targets, the bovine target MDBK was efficiently lysed by NK cells from all donors. Due to interspecies sequence differences, mouse and human target cells might express few ligands efficiently recognized by bovine activating NK receptors.

In the redirected lysis assay, mimicking a ligand for NKp46 induced vigorous cytotoxicity even at low effector to target cell ratios. Thus, in animal 1, ~70% specific ^{51}Cr release from P815 targets was obtained at a 25:1 ratio without antibody, whereas the same level of cytotoxicity was observed at a 4:1 ratio following incubation with AKS1. The lower cytotoxicity in the spontaneous lysis situation probably indicates that only a subset of NK cells express the appropriate receptors that allow killing, whereas all cells expressed NKp46 and could be activated via the AKS1 mAb. Lysis of the FcR$^-$ target MDBK was inhibited by incubation with AKS1, suggesting that the mAb blocked interaction with a ligand on the target cells that was important for induction of cytotoxicity. Our data do not exclude that cross-linking of NKp46 alone may be sufficient to activate the cytolytic machinery, but further studies investigating a larger array of NK receptors on single clones will be required to address this question.

The bovine IL-2-activated NK cells expressed CD8 mainly in the heterodimeric (α/β) form. This contrasts to the situation in man and rat where NK cells predominantly express the CD8α homodimer [26, 27], whereas

murine NK cells do not express CD8 [22]. The functional role of CD8 on NK cells is not clear. In this study, preincubation with anti-CD8 mAb had no effect on the level of cytotoxicity towards FcR^- or FcR^+ targets (Fig. 5B, C).

We have described a method whereby bovine NK cells can be purified and cultured. In contrast to bovine T cells that proliferate in response to human recombinant IL-2 [28], bovine IL-2 was required for the culture of bovine NK cells. Bovine $NKp46^+$ cells could also to some extent be expanded in supernatant from Con A-stimulated bovine $CD4^+$ T cells (data not shown).

This initial characterization of NK cells in cattle and the availability of an mAb specifically reacting with bovine NK cells will hopefully facilitate future studies of innate immunity in cattle.

4 Materials and methods

4.1 Generation of an anti-NKp46 mAb

An expression construct containing the extracellular region of bovine NKp46 [13] at the N-terminal side of the Fc region of mouse IgG2b was constructed. Briefly, the NKp46 signal sequence and extracellular region (nucleotides 4–774) was amplified by PCR using *PfuTurbo* DNA polymerase (Stratagene, La Jolla, CA) and gene-specific primers including HindIII and BamHI restriction sites. The PCR product was purified by agarose gel electrophoresis, incubated with Taq DNA polymerase (Promega, Madison, WI), cloned into pCR2.1 TOPO vector (Invitrogen, San Diego, CA), released by HindIII and BamHI digestion (New England Biolabs, Beverly, MA) and ligated into a mammalian expression vector containing the hinge, C_H2 and C_H3 regions of the mouse IgG2b gene (obtained from H. C. Aasheim, The Norwegian Radium Hospital, Oslo, Norway). Of this construct, 20 μg was mixed with 120 μl Lipofectamine (Invitrogen) in 2.8 ml Opti-MEM (Invitrogen) and incubated for 20–30 min at room temperature; 11.2 ml Opti-MEM were then added and the mixture transferred to a 160-cm^2 flask containing an ~70% confluent layer of 293T cells. After 6 h, 14 ml of complete RPMI 1640 medium/20% FCS (Invitrogen) was added. The cells were then incubated for 18 h, washed three times in Opti-MEM or PBS and finally cultured in serum-free AIM-V medium (Invitrogen) for 4 days. bNKp46-mFcγ2b fusion protein was purified from the culture supernatant on a protein G column (Amersham Biosciences, Little Chalfont, GB) according to the manufacturer's instructions. Female young adult BALB/c mice were immunized by four intraperitoneal injections of 100 μg fusion protein in Freund's complete (first injection) or incomplete adjuvant (following two injections, Invitrogen) or in PBS (final injection), and spleen cells were fused with NS-0 cells by conventional techniques. Single-clone hybridoma supernatants were screened by flow cytometry (see below) for reactivity against 293T cells transiently transfected with an expression construct containing the entire coding region of NKp46 and a FLAG tag in the N-terminal end (generated by amplifying the coding region of NKp46 without the signal sequence (nucleotide 88 to 951) by PCR and inserting it into pFLAG-CMV-1 (Sigma-Aldrich, St. Louis, MO). One specifically reacting hybridoma clone, with a staining pattern similar to that of anti-FLAG antibody M2 (Sigma-Aldrich), was subcloned twice and named AKS1.

4.2 Western blotting and immunoprecipitation

293T cells transfected with the FLAG-NKp46 construct or empty vector were harvested 48 h post transfection and lysed with 1% Igepal CA-630 (Sigma-Aldrich) in lysis buffer [25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF and protease inhibitor mix (Sigma-Aldrich)] at 4°C. For immunoprecipitations, IL-2-activated NK cells were surface-biotinylated as follows: cells were washed three times in PBS pH 8.0, incubated for 30 min at room temperature with 0.5 mg/ml NHS-biotin (Sigma-Aldrich) in PBS pH 8.0 at 25×10^6 cells/ml, then washed three times in PBS pH 8.0. Biotinylated cells were lysed in 1% Triton X-114 (Sigma-Aldrich) as described [29]. The membrane-enriched lysate fraction from 7×10^6 cells (~20 μl) was diluted in 400 μl lysis buffer (as above) containing 0.5% digitonin (Calbiochem, San Diego, CA), and precleared with 30 μl protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) precoated with irrelevant antibody (5 μg mAb W6/32 [30] per 100 μl beads) three times at 4°C; twice for 1 h, and the third step overnight. Precleared lysate was then immunoprecipitated for 2 h at 4°C with 30 μl beads precoated with 5 μg AKS1 mAb, and the beads were washed three times by centrifugation for 4 min at 2,300×g and resuspension in lysis buffer/0.5% digitonin.

Protein samples were diluted in SDS sample buffer, boiled for 2 min, separated by SDS-PAGE, and transferred to PVDF membrane by semi-dry transfer. Membranes were blocked with blocking buffer (TBS/0.05% Tween 20/3% BSA) and incubated with rabbit anti-FLAG antibody diluted 1:1,000 (Sigma-Aldrich) or AKS1 at 4 μg/ml in blocking buffer for 2 h at room temperature. Membranes were washed six times in TBS/0.05% Tween 20 and incubated with peroxidase conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, PA) in blocking buffer. Detection of secondary Ab was with SuperSignal West Pico substrate (Pierce Biotechnology, Rockford, IL) and a high-performance CCD camera (Kodak ImageStation 2000, Eastman Kodak, Rochester, NY).

4.3 Culture of bovine NK cells

PMBC were separated from blood collected in EDTA tubes by Lymphoprep (Axis-Shield, Oslo, Norway) gradient centrifugation (1,150×g, 20 min) and washed twice in PBS with 2 mM EDTA. Subsequently, 3×10^8 cells in 6 ml PBS with

2 mM EDTA and 0.5% BSA were added to 18 μg AKS1 mAb and incubated at 4°C for 30 min. After washing twice, 24×10^6 immunomagnetic beads coated with an anti-mouse pan IgG mAb (Dynal, Oslo, Norway) were added and incubated at 4°C for 30 min with shaking, fixed in a magnet and washed three times in PBS with 0.5% BSA. Positively selected cells were cultured in a six-well tray in 3 ml of RPMI 1640 supplemented with 60 μg/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, nonessential amino acids, 50 μM 2-mercaptoethanol, 10% FCS (all Invitrogen) and recombinant bovine IL-2 (10 biological U/ml final concentration) obtained by expression in COS cells. After 24–48 h, magnetic beads were collected and the cells were transferred to new wells. Additional medium was added when necessary. The IL-2 concentration in added medium was reduced to 4 or 2 biological U/ml after 5–6 days when cultures were proliferating.

4.4 Cytospin preparations

IL-2-activated NKp46$^+$ cells (5×10^4 in 50 μl) were centrifuged onto poly-L-lysine-coated glass slides at 80×g for 6 min, immediately fixed in methanol for 5 min, air dried, and stained with a 1:20 dilution of Giemsa stain (Sigma-Aldrich) in water for 20–30 min, and washed with water buffered to pH 7.0 (3 mM sodium phosphate). Blood NK cells were positively selected by AKS1 antibody and CELLection pan mouse IgG magnetic beads (Dynal), released by DNase treatment, washed twice in RPMI 1640/10% FCS prior to cytospin preparation and stained as described above.

4.5 Flow cytometry

Two-color flow cytometric analysis of cell surface receptors was used to determine the expression of leukocyte receptors on NKp46$^+$ cells in peripheral blood. Blood from eight 6–12-month-old cattle of the Norwegian Dairy Cattle breed was lysed hypotonically, and 10^6 leukocytes were incubated with AKS1 at 1 μg/ml in PBS with 1% BSA and 10 mM NaN_3 and one of the following antibodies: CD4 (IL-A11, 10 μg/ml), CD8α (BAQ111A, 5 μg/ml), CD2 (MUC2A, 10 μg/ml), CD25 (LCTB2A, 5 μg/ml), TCR1 (GB21A, 5 μg/ml), WC1 (B7A1, 10 μg/ml), MHC class II (H42A, 0.5 μg/ml), B cells (BAQ44A, 10 μg/ml), granulocytes [CH138A, 5 μg/ml, all from Veterinary Medical Research Diagnostics (VMRD), Pullman, WA] and CD14 (TÜK4, 0.6 μg/ml, DAKO, Glostrup, Denmark). Subtype-specific secondary antibodies conjugated with allophycocyanin (anti-IgG1 mAb, BD Biosciences, San Jose, CA) or FITC (goat anti-mouse Ab, against all other isotypes, Southern Biotech, Birmingham, AL) were used. Control stainings with secondary antibodies only and isotype control antibodies were performed. Cells were fixed by adding FACS lysing solution (BD Biosciences) prior to analysis. Gates were set to include mononuclear cells or the total leukocyte population in the granulocyte marker stainings. A total of 10,000 gated cells were analyzed.

Tissue samples from thymus, spleen, mesenterial and retropharyngeal lymph nodes, liver and lungs were collected from five animals of the Norwegian Dairy Cattle breed at 6–24 months of age at the abattoir, kept on ice and homogenized as previously described [31]. The surface expression of the leukocyte markers described above, including CD8β (BAT82A, 10 μg/ml) and CD3ε chain (MM1A, 10 μg/ml, both from VMRD) was also analyzed on IL-2-activated NK cells from five different animals.

4.6 Cytotoxic activity of bovine NK cells

The cytotoxic activity of IL-2-activated bovine cells against the bovine kidney cell line MDBK [32], the murine tumor cell lines YAC-1 [33] and P815 [34] and the human leukemic cell line K562 [35] was tested in a standard ^{51}Cr-release assay: target cells were maintained in complete RPMI 1640/10% FCS. Target cells (5×10^6 cells) were incubated with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham Biosciences) in 1 ml medium at 37°C for 1 h and were washed three times in PBS/2% FCS. Twofold dilutions of effector cells (6–14 day NK cell cultures) were added to 1×10^4 ^{51}Cr-labeled target cells in 96-well round-bottom microtiter plates and incubated at 37°C for 4 h. The supernatant was harvested and radioactivity counted on a gamma-counter. Specific ^{51}Cr release was calculated on the basis of the ratio [(sample release – spontaneous release)/(total release – spontaneous release)]. The results were expressed as the median of three parallel samples. In blocking experiments with MDBK targets and in redirected lysis assays of the FcγR$^+$ target cell line P815 the effector cells were preincubated for 20-30 min with 1 μg/ml mAb (all IgG1) against NKp46 (AKS1), CD8 (CACT80C) or CD2 (BAQ95A, both from VMRD). MDBK grows as a monolayer, and was released by non-enzymatic treatment (Cell dissociation solution, Sigma) prior to the ^{51}Cr labeling.

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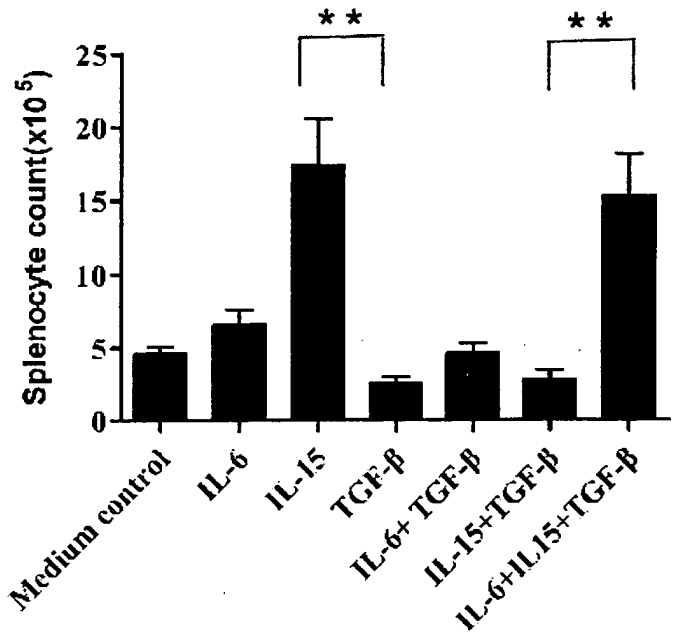

Fig 1. The splenocytes count. To measure the ability of IL-2, IL-6, IL-15 or TGF-β to induce proliferation to splenocytes, splenocytes without monocytes were incubated with IL-2, TGF-β, IL-6, IL-15, TGF-β and IL-6, TGF-β and IL-15, or TGF-β and IL-6 /IL-15, at the same concentrations, was added again on day 3. For 6 days later, the splenocytes were count. Results are the mean ± the standard error (bars)(** $p<0.01$).

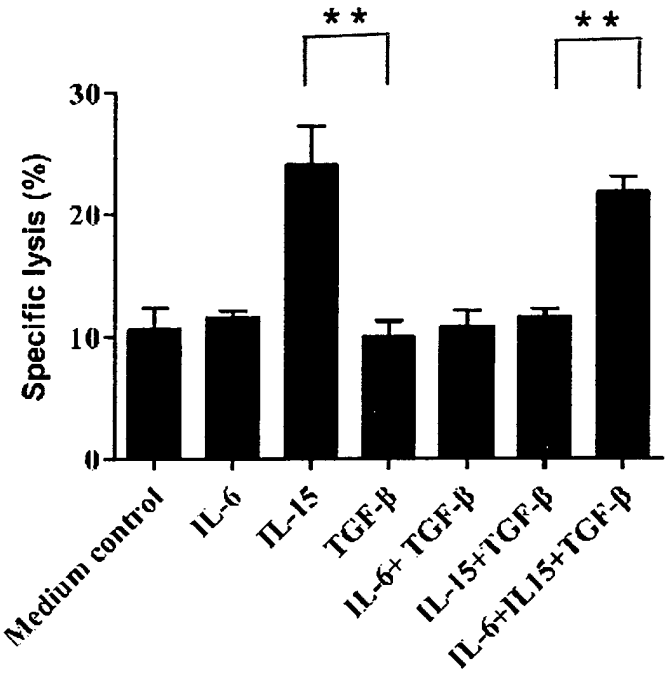

Fig 2. Treatment with IL-6/IL-15 reversed TGF-β inhibition of LAK cytotoxicity. Fresh splenocytes without monocytes contamination were cultured for 6 days in IL-2 (0.5 μg /ml), TGF-β (0.01 μg /ml), IL-6 (0.04 μg / ml), IL-15 (0.2 μg /ml), TGF-β (0.01 μg /ml) and IL-6 (0.04 μg / ml), TGF-β (0.01 μg /ml) and IL-15 (0.2 μg /ml), or TGF-β (0.01 μg /ml) and IL-6 (0.04 μg /ml)/IL-15 (0.2 μg /ml). On day 6, splenocyte cytotoxicity to YAC-1 cells, at an E/T ratio of 13:1, was measured. Data are the results of triplicate samples from three independent experiments. Results are the mean ± the standard error (bars) (** $p<0.01$).